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Application No.: 10/040281

Docket No.: BGNB129CP2DV2CN

**REMARKS**

Claims 25, 27, and 29-38 were pending in the application. Claims 25, 27, 29-32 and 34-38 have been canceled herein. Cancellation of these claims should in no way be construed as an acquiescence to any of the Examiner's rejections. The cancellation of these claims is being made solely to expedite prosecution of the above-identified application. Applicants reserve the option to further prosecute the same or similar claims in the instant or in another patent application. Claim 33 has been amended. Support for amended claim 33 can be found in claim 29 as previously pending, and throughout the specification as filed. New claims 39-42 have been added. Support for new claims 39-42 can be found in pending claim 33. No new matter has been added.

The Examiner has rejected pending claim 33 under the first paragraph of 35 U.S.C. 112. The Examiner states that a review of the prior art "does not indicate that any one of [the cytokines cited as analogous to LT- $\beta$ , nor forms of LT- $\beta$  presently claimed] whether alone or in complexes or in soluble form or antibodies direct[ed] unto can be used as in the treatment of all the diseases as claimed." The Examiner additionally states that "[o]ne of skill in the art would be led to perform undue experimentation to determine the applicability of treating a disease state with the polypeptides and antibodies recited in the instant claims."

Applicants respectfully traverse this rejection. In the present application, Applicants teach a role for LT- $\beta$  and complexes including LT- $\beta$  in processes in which cells such as lymphocytes kill tumor cells (refer, e.g., to page 35, lines 13-16 of the instant specification). Applicants describe additional uses of LT- $\beta$  in cell- and vaccine-based treatments of neoplasia in the section spanning page 35, line 13 to page 36, line 10. Applicants additionally teach that various forms of LT- $\beta$  are useful in treatments requiring cytolytic activities, such as inhibition of tumor cell or neoplasia growth (for example, refer to page 9, lines 14-20; Balkwill et al. (1986) *Cancer Res.* **46**, 3990; Palladino et al. (1987) *J. Immunol.* **138**, 4023-4032). Thus, Applicants provide sufficient teachings to enable one skilled in the art to use various forms of LT- $\beta$  in treatment of neoplasia without undue experimentation.

Post-filing date evidence in support of the use of various forms of LT- $\beta$  in the treatment of neoplasia can be found in Applicants' own work. Various forms of LT- $\beta$  have been shown to

inhibit growth of a variety of tumor cells. Specifically, forms of LT- $\beta$  in various forms of complex with LT- $\alpha$  were demonstrated to be cytotoxic to a variety of tumor cells, including human adenocarcinoma cell lines HT-29, WiDr, and MDA-MB-468, as well as to WEHI-164 cells (recognized as TNF-responsive) (refer to Browning *et al.* (1996) *J. Exp. Med.* **183**, 867-878; and to Figure 4 of Mackay *et al.* (1997) *J. Immunol.* **159**, 3299-3310 (copies enclosed)). These results confirm Applicants' teachings of the use of various forms of LT- $\beta$  as a therapeutic agent for the treatment of neoplasia. Thus, in view of Applicants' teachings, one of skill in the art can practice the claimed method without undue experimentation.

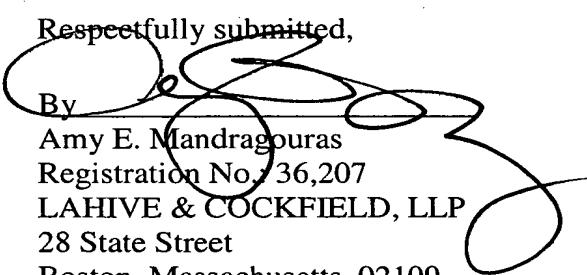
In view of the above amendment, Applicants believe the pending application is in condition for allowance.

Applicants believe no fee is due with this statement. However, if a fee is due, please charge our Deposit Account No. 12-0080, under Order No. BGNB129CP2DV2CN from which the undersigned is authorized to draw.

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Respectfully submitted,

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# Cytotoxic Activities of Recombinant Soluble Murine Lymphotoxin- $\alpha$ and Lymphotoxin- $\alpha\beta$ Complexes

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Human lymphotoxin- $\alpha$  (LT $\alpha$ ) is found in a secreted form and on the surface of lymphocytes as a complex with a second related protein called lymphotoxin- $\beta$  (LT $\beta$ ). Both secreted human LT $\alpha$  and TNF have similar biological activities mediated via the TNF receptors, whereas the cell surface LT $\alpha\beta$  complex binds to a separate receptor called the LT $\beta$  receptor (LT $\beta$ R). The murine LT $\alpha$  and LT $\beta$  (mLT $\alpha$  and mLT $\beta$ ) proteins have never been characterized. When recombinant mLT $\alpha$  was produced by either of several methods, the protein had a very low specific activity relative to that of human LT $\alpha$  in the conventional WEHI 164 cytotoxicity bioassay. The weak activity observed was inhibited by a soluble murine TNF-R55 Ig fusion protein (mTNF-R55-Ig), but not by mLT $\beta$ R-Ig. Coexpression of both mLT $\alpha$  and a soluble version of mLT $\beta$  in insect cells led to an LT $\alpha\beta$  form that was cytotoxic in the WEHI 164 assay via the LT $\beta$ R. To determine whether natural mLT $\alpha$ -like forms with cytotoxic activity comparable to that of secreted human LT $\alpha$  were secreted from primary spleen cells, splenic lymphocytes were activated in various ways, and their supernatants were analyzed for cytotoxic activity. Using specific Abs to distinguish between mTNF and mLT, a TNF component was readily detected; however, there was no evidence for a secreted mLT $\alpha$  cytotoxic activity using this assay. Combined, these observations suggest that secreted mLT $\alpha$  may not play a role in the mouse via interactions with TNF-R55, and the ramifications of this hypothesis are discussed. *The Journal of Immunology*, 1997, 159: 3299–3310.

Like TNF, lymphotoxin- $\alpha$  (LT $\alpha$ )<sup>3</sup> is found in both secreted and membrane-associated forms in humans, yet unlike TNF, surface LT $\alpha$  is tethered to the surface via complexation with a related protein called lymphotoxin- $\beta$  (LT $\beta$ ) (1–3). Signaling in this system proceeds via either of two pathways, the interaction of TNF or LT $\alpha$  with the TNF receptors (TNF-R) and the binding of LT $\alpha\beta$  to the LT $\beta$  receptor (LT $\beta$ R) (4, 5). Progress in the LT field has been slower than that for TNF, since, historically, it was assumed that the function of secreted LT $\alpha$  was identical with that of TNF, and the nature of the LT $\alpha\beta$  complex was only recently uncovered. Secondly and perhaps more fundamentally, other than RNA expression studies, very little is known about the biochemistry of the mouse LT forms (6–12). Without murine models and reagents, it has been difficult to study the immunologic role of LT. Recently, the disruption of both LT genes in mice has contributed substantially to an understanding of the role of LT in

controlling lymphoid development and architecture (13, 14). Now, given the emergence of this unique area of immunology, it is especially important to delineate between the roles of soluble and surface LT forms in the developmental and physiologic processes.

To facilitate the biochemical analyses, we have taken the approach of preparing recombinant mLT $\alpha$  and have re-engineered the normally membrane-bound LT $\alpha\beta$  into a soluble version. Soluble human trimers with LT $\alpha$ 1 $\beta$ 2 and LT $\alpha$ 2 $\beta$ 1 stoichiometry have been made and characterized (3). These trimers are stable and biologically active. Unlike TNF, the soluble heteromeric LT forms lack proinflammatory activity (15). LT $\alpha$ 1 $\beta$ 2 can induce the death of several adenocarcinoma tumor lines, and this effect is mediated by the LT $\beta$ R (16). When this technology was duplicated to generate mLT forms, the pure soluble mLT $\alpha$  trimer lacked the classical potent cytotoxic activity characterizing hTNF and hLT $\alpha$ . When coexpressed with LT $\beta$ , the resultant heteromeric form is cytotoxic via the LT $\beta$ R pathway. Activated splenic lymphocytes should secrete soluble mLT $\alpha$ , yet no evidence for mLT $\alpha$  cytotoxic activity was found in the cell supernatants. These results suggest the tantalizing proposition that soluble LT $\alpha$  may not signal through the TNF-R55, raising several issues with the interpretation of the various genetic knockout phenotypes.

## Materials and Methods

### Soluble receptor-Ig fusion proteins

The receptor-Ig fusion proteins used in this study were murine versions of both TNF-R55 and LT $\beta$ R coupled to the hinge, CH2, and CH3 domains of hIgG1 as described in the accompanying publication. The control fusion protein, hLFA-3-Ig has been described previously (17).

### Recombinant cytokines

Recombinant *Escherichia coli*-derived mTNF was provided by Biogen and later purchased from Genzyme (Cambridge, MA). The two preparations

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<sup>3</sup> Abbreviations used in this paper: LT $\alpha$ , lymphotoxin- $\alpha$  (originally called tumor necrosis factor- $\beta$ ); LT $\beta$ , lymphotoxin- $\beta$ ; TNF-R, tumor necrosis factor receptor; LT $\beta$ R, lymphotoxin- $\beta$  receptor; mLT $\alpha$  and mLT $\beta$ , murine lymphotoxins; TNF-R55, 55- to 60-kilodalton tumor necrosis factor receptor; h, human; LT $\beta$ R-Ig, LT $\beta$  receptor-immunoglobulin fusion protein; TNF-R55-Ig, TNF-R55 immunoglobulin fusion protein; mTNF, murine tumor necrosis factor; i.m., intramuscularly.

had similar bioactivities. The vectors for mLT $\alpha$  expression were constructed using a mLT $\alpha$  cDNA plasmid pMV-SM7A from Dr. Nancy Ruddle that extended through a poly(A) tail and encoded for a mLT $\alpha$  protein that initiated at the methionine closest to the transmembrane region. The mLT $\alpha$  insert was excised by digestion with EcoRI and BamHI, and the 1.5-kb fragment was subcloned into pNN01 (a pUC19 derivative with a modified polylinker) such that the LT $\alpha$  insert could be excised from pNN01 by digestion with BamHI alone. This BamHI fragment was then cloned into the BamHI sites of pBlueBac3 (Invitrogen, San Diego, CA), yielding the vector CH211, which was used to generate recombinant baculovirus. The entire BamHI insert was also cloned into the BamHI site of pCDNA3, yielding CH242. A nonmethylated DNA preparation of CH211 was digested with SmaI and StuI, and the 650-bp fragment containing the coding region of mLT $\alpha$  was cloned into the EcoR5 site of pCDNA3 (Invitrogen), yielding the vector CH241. The vector CH242 was digested with HindIII and NotI, and the 1.5-kb LT $\alpha$  insert was gel purified and cloned into HindIII- and NotI-digested pCDM8 (CH243). A mLT $\alpha$  expression plasmid with a Kozak consensus translation initiation sequence and a deleted 3' untranslated region was constructed by PCR with oligos 5'-GAAT TCGCGGCCGCCACCATGGCACTGCTCGGGCGTC-3' and 5'-GAATT CGCGGCCGCCATCAGTGCAAAGGCTCC-3'. PCR was conducted using *Pfu* polymerase (Stratagene, La Jolla, CA) and the supplied buffer, 12.5 nmol dXTTP, 30 pmol of each primer, 100 ng of plasmid CH211, and 10% DMSO (final concentration) in 100  $\mu$ l. After initial incubation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min were conducted. The PCR product was isolated, digested with NotI, and cloned into pCDM8, yielding CH244.

To express mLT $\beta$  forms, a soluble *myc*-tagged mLT $\beta$  construct was created in the baculovirus vector pBlueBac3. A PCR product containing a portion of the extracellular region of mLT $\beta$  was amplified from a cDNA clone SP4-3 (8) using the phosphorylated primer 5'-GGGGTCAACAG CTGCAAAGGGG-3' and a 3' primer containing a HindIII site (5'-TAC GAAAGCTTGGTCCCGGACATCACGAT-3'). The reaction contained 20 mM Tris-Cl pH 8.8; 10 mM KCl; 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 2 mM MgSO<sub>4</sub>; 0.1% Triton X-100; 100  $\mu$ g/ml BSA; 10% DMSO; 0.2 mM dNTPs; 10 ng template; 100 ng primer; and 1.25 U *Pfu* polymerase (Stratagene) for 25 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min with a 5-min extension at 72°C. A fragment containing the vascular cell adhesion molecule-1 signal sequence and the *c-myc* tag was amplified from the plasmid smycp33 (5), using a 5' primer with a BamHI site (5'-CATTGGATC CCACTGGCTTCAGGAGC-3') and the 3' primer (5'-CAGGTCTTCT TCAGAGATGAGTT-3') as above, but without the DMSO, for 25 cycles at 94°C for 30 s, 65°C for 1 min, and 72°C for 2 min followed by a 5-min extension at 72°C. The fragments were digested for about 20 h, low melt gel purified, and then ligated into BamHI- and HindIII-linearized pBlueBac3, resulting in the vector CA107. The recombinant baculovirus was produced as described previously (3). To express soluble, *myc*-tagged mLT $\beta$  in COS cells, the BamHI+HindIII fragment from the baculovirus construct was cloned into a pNN03 to apply flanking NotI sites. The NotI fragment from this subclone was ligated into the NotI site of pCDM8, resulting in the vector CA113.

To express LT forms in insect cells, a suspension culture of High Five cells (Invitrogen) was infected with mLT $\alpha$  baculovirus at a multiplicity of infection of 3 in 125-ml Spinner flasks in SF900 II serum-free medium (Life Technologies, Gaithersburg, MD). The infected cells were incubated for 48 h at 28°C; conditioned medium was harvested as previously described (3); and 1% FBS, 1 mM EDTA, 0.02% sodium azide, and 100  $\mu$ M PMSF were added before sterile filtering the medium through a 0.45- $\mu$ m pore size membrane. The mLT $\alpha$  heteromeric complexes were produced by the simultaneous coinfection of High Five cells with mLT $\alpha$  and mLT $\beta$  baculovirus at multiplicities of infection of 3 and 7, respectively. After various time points, aliquots were taken for activity analysis, or at 48 h conditioned medium was harvested as described above for purification of the ligands.

Mouse LT $\alpha$  was expressed transiently in COS cells following electroporation of  $1 \times 10^7$  cells with 20  $\mu$ g of CH241, CH242, CH243, or CH244 mixed with 380  $\mu$ g of sonicated salmon sperm DNA. After 4 h the medium was replaced, and aliquots were taken at various times thereafter for analysis.

#### Purification of mLT $\alpha$ and mLT $\alpha$ $\beta$ forms

To analyze murine or human LT $\alpha$  expression, 1 ml of either uninfected or mLT $\alpha$  baculovirus-infected High Five cell-conditioned medium was precipitated with 5  $\mu$ g of human TNF-R55-Ig and protein A-Sepharose Fast Flow (Pharmacia, Piscataway, NJ). The beads were washed four times with PBS/0.3% Tween-20 and eluted with SDS-PAGE nonreducing sample buffer. The precipitations were then run on a 10 to 20% SDS-PAGE gel.

Purification of murine LT $\alpha$  trimer was performed by affinity chromatography of conditioned medium with human TNF-R55-Ig coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) as previously described (3). The bound protein was washed with 10 vol of PBS, eluted with 25 mM sodium phosphate buffer, pH 2.8/100 mM NaCl, and immediately neutralized with a 0.5-M sodium phosphate buffer, pH 8.6.

Conditioned medium from insect cells coinfecting with mLT $\alpha$  and mLT $\beta$  baculovirus was loaded onto a mLT $\beta$ -Ig coupled to a cyanogen bromide-activated Sepharose affinity column and eluted as described for mLT $\alpha$ . The eluted material is referred to as generic mLT $\alpha$  $\beta$ . The generic mLT $\alpha$  $\beta$  was further fractionated in some experiments by repeated passage over an hTNF-R55-Ig affinity column to enrich the preparation in mLT $\alpha$  $\beta$ . The eluate from the hTNF-R55-Ig column was mLT $\alpha$  $\beta$ . Protein was analyzed for purity by SDS-PAGE and quantitated by absorbance at 280 nm using 1 OD = 1 mg/ml.

#### Analytical methods

Affinity, gel exclusion, ion exchange, and C<sub>4</sub> reverse phase chromatography as well as N-terminal amino acid sequencing were performed as described for the analysis of the human LT forms (3). BIACore analyses were conducted with receptor-Ig fusion proteins immobilized on N-hydroxysuccinimide-activated sensor chips. Sensor chips were loaded to the 2000 resonance units range, and ligands were passed over the bound receptor in the BIACore buffer system.

#### Abs and ELISA determination

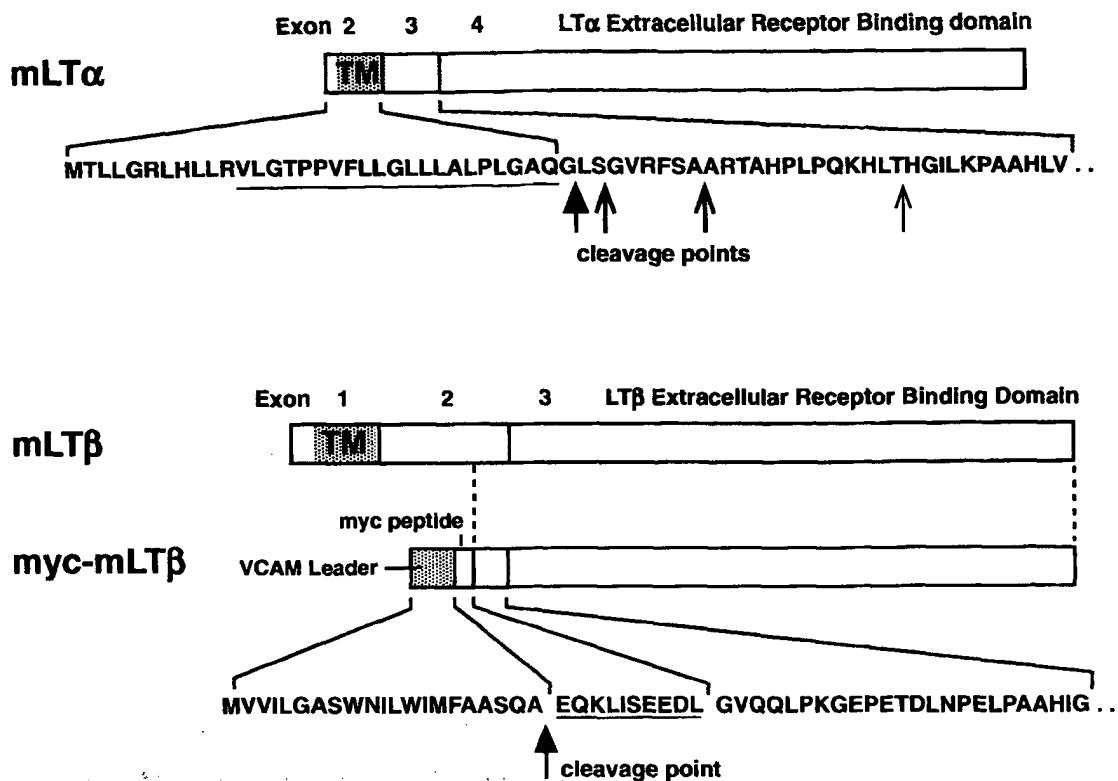
The antagonistic anti-mTNF-R55 and anti-mTNF-R75 mAbs, 55R-170, and TR75-32 were purchased from Genzyme, and the hybridoma secreting the control anti-KLH Armenian hamster Ig, Ha4/8, was a gift from Dr. D. Mendrick. The rat anti-mTNF MP6-XT22 (Life Technologies and subsequently PharMingen (San Diego, CA)) and hamster anti-mTNF TN3-19.12 mAb (Genzyme) Abs were purchased. The origin of the hamster anti-mLT $\alpha$  and mLT $\beta$  mAbs has been described in a separate publication (17a). A goat polyclonal anti-mLT $\alpha$  antiserum was prepared following i.m. immunization with 350  $\mu$ g of baculovirus-expressed mLT $\alpha$  in CFA followed by two s.c. and one i.m. booster injections of about 150  $\mu$ g each in IFA. Previously we had found that intralymph node injection of hLT $\alpha$  in CFA in rabbits led to a good neutralizing Ab response (18). When rabbits were immunized in the same manner with mLT $\alpha$ , the animals did not mount a high titer response.

To create anti-mLT $\beta$  Abs, an Armenian hamster (Dr. Yerganian, Cytogen, Boston, MA) was immunized via i.p. injection with approximately 50  $\mu$ g of mLT $\beta$ -Ig coupled to protein A beads (Pharmacia) without adjuvant and boosted once under the same conditions. The hamster was boosted 1 mo later via the same route with 100  $\mu$ g of mLT $\beta$ -Ig coupled to protein A beads, then again 3 days before fusion, and finally again with 100  $\mu$ g of uncoupled soluble mLT $\beta$ -Ig i.v. 1 day before fusion. Spleens were fused, and hybridomas were screened by ELISA methods and by FACS for binding to L929 cells and for the ability to block the binding of surface LT-positive PMA-activated IL-23 cells to receptor-coated plates. ELISA assays for binding inhibition, IL-23 adhesion assay, FACS assay for receptor binding, and BIACore epitope mapping were conducted as described previously (16).

Recombinant mLT $\alpha$  was quantitated by coating Immulon 2 plates (Dynatech Laboratories, Chantilly, VA) with hamster anti-mLT $\alpha$  mAb AFB3. Plates were washed, blocked with 2% BSA in PBS, and then incubated with varying concentrations of mLT $\alpha$  or mTNF. Bound mLT $\alpha$  was probed with a 1/5,000 dilution of goat anti-mLT $\alpha$  antiserum, probed with a horseradish peroxidase-coupled donkey anti-goat antiserum at a 1/10,000 dilution (Jackson ImmunoResearch Laboratories, West Grove, PA), and developed using tetramethylbenzidine methodology. The derivation of the anti-mLT $\alpha$  mAb is described in the accompanying report (17a). A small level of background binding (<0.2 OD units) was observed when mLT $\alpha$  was added to a plate coated with a control hamster anti-TNP mAb, and this background optical density (0.2 OD units at 1  $\mu$ g/ml) was subtracted from the raw data to yield the binding curve shown in Figure 2. No appreciable background binding was noted with mTNF.

#### Secretion of cytotoxic activity from activated mouse spleen cells

Spleens were isolated from BALB/c mice, dissociated by mincing and trituration, and RBC were removed by lysis with Gey's solution. The remaining cells were cultured at  $3 \times 10^6$  cells/ml in RPMI 1640 with 10% FBS (HyClone defined, Logan, UT), glutamine, and 10 mM HEPES buffer, pH 7.5, with various combinations of 1  $\mu$ g/ml staphylococcal enterotoxin



**FIGURE 1.** Schematic diagrams of the LT proteins and the constructs used in this study. The upper half of the figure shows the mLT $\alpha$  protein and its amino acid sequence. The putative transmembrane domain/leader sequence is underlined, and cleavage points are noted, with the intensity of the arrow indicating roughly the prominence of the species following insect cell secretion. In the lower half, the natural mLT $\beta$  protein is shown, illustrating the portion used to form the artificial myc-mLT $\beta$  secretion construct. The myc tag is underlined, and the arrow indicates the actual cleavage point.

B (Sigma Chemical Co., St. Louis, MO), 10 ng/ml PMA, 1  $\mu$ g/ml hamster anti-CD3- $\epsilon$  mAb 145-2C11 (Boehringer Mannheim, Indianapolis, IN), or 10 ng/ml human rIL-2 provided by Biogen. After a 24-h incubation at 37°C, supernatants were collected and assayed immediately for activity in a 1-day WEHI 164.13 cytotoxicity assay. Supernatants were stored frozen for further analyses.

The 1-day cytotoxicity assay with WEHI 164.13 cells was performed basically as previously described (3). Briefly, cells were plated at 20,000 cells/well in flat 96-well microplates in a volume of 100  $\mu$ l. The next day, 50  $\mu$ l of the medium was removed, and 50  $\mu$ l of the spleen cell supernatant at various dilutions was added along with 10  $\mu$ l of 100  $\mu$ g/ml cycloheximide. On the following day the viable cell mass was quantitated as previously described (3). The 3-day antiproliferative assay has been described previously (3).

## Results

### Characterization of recombinant mLT $\alpha$

Pure recombinant mLT $\alpha$  was prepared using a baculovirus/insect cell expression system previously employed to produce active forms of human LT $\alpha$  and LT $\alpha\beta$  complexes (3, 19, 20). The expression of mLT $\alpha$  was monitored by immunoprecipitation with either human or mouse TNF-R55-Ig and direct visualization by SDS-PAGE. Insect cell-derived mLT $\alpha$  was expressed at the 10 to 20  $\mu$ g/ml level, and following purification on a hTNF-R55-Ig affinity column, it appeared as a 20-kDa species with minor forms at about 19 and 17 kDa (data not shown). The preparations were >95% pure based on SDS-PAGE and C<sub>4</sub> reverse phase HPLC. The constructs and processing sites in both mLT $\alpha$  and mLT $\beta$  are diagrammed in Figure 1. The processed species of mLT $\alpha$  are similar in size to those of human LT $\alpha$  produced in the same system. N-terminal amino acid sequence analysis of each of the bands

showed that the primary processing site was after Gly<sup>32</sup>, with additional cleavage events appearing after Ser<sup>34</sup>, Ala<sup>40</sup>, and Thr<sup>53</sup>. The primary form is a 169-amino acid species with a predicted size of 18.54 kDa, and the observed size of about 20 kDa suggests that this material was glycosylated. As most members of the TNF family are retained on the membrane surface, i.e., they are not secreted, it was not clear whether mLT $\alpha$  would be secreted. A previous study had shown that only very weak cytotoxic activity could be detected in the supernatants following COS cell transfection, suggesting possible poor processing (21). The expression levels observed here in both insect and COS cell (see below) systems were comparable with those observed for the human constructs; therefore, it is likely that natural mLT $\alpha$  will be found in a secreted form. Thus, mLT $\alpha$  appears to be very similar to hLT $\alpha$ , since the primary cleavage event occurs at the equivalent amino acids in both mouse and human LT $\alpha$ , and the predominant N-terminal cleavage forms are similar in both cases (22). The insect cell-derived mLT $\alpha$  was insoluble at high concentrations. Typically, at concentrations >0.2 mg/ml, some precipitation was noticed during elution off the affinity column as well as at neutral pH when concentration was attempted by ultrafiltration using a 10-kDa cut-off membrane.

Due to the conservation of amino acid residues in the framework regions responsible for trimerization, it is expected that all members of the TNF family of ligands will be trimers (2). Gel exclusion sizing chromatography analysis of mLT $\alpha$  showed that the molecule was trimeric when chromatographed on a TSK G3000 column (TosoHaas, Montgomery, PA) in the presence of 0.5 M NaCl (data not shown). At physiologic salt conditions, it appeared that mLT $\alpha$

Table I. An analysis of the cytotoxic activity of recombinant murine and human LT $\alpha$  expressed in different systems

Source	Assay System <sup>a</sup>	LT $\alpha$ Protein (ng/ml)	Cytotoxicity Activity (U/ml) <sup>b</sup>	Specific Activity (U/mg)
Cos cell Expression <sup>c</sup>				
Vector control	L929		<1	
mLT $\alpha$	L929	~20–50 <sup>d</sup>	<1	<2.0 × 10 <sup>4</sup>
hLT $\alpha$	L929	~100 <sup>d</sup>	13,100	~1.3 × 10 <sup>6</sup>
mLT $\alpha$	WEHI	45 <sup>e</sup>	<1	<2.2 × 10 <sup>4</sup>
Insect Cell Expression <sup>f</sup>				
Media alone	L929		0	
mLT $\alpha$	L929	~10,000 <sup>d</sup>	5	~1 × 10 <sup>3</sup>
hLT $\alpha$	L929	~20,000 <sup>d</sup>	25,600	~1.2 × 10 <sup>6</sup>
mLT $\alpha$	WEHI	3,000 <sup>e</sup>	8	5 × 10 <sup>3</sup>
Affinity Purified				
mLT $\alpha$	L929	— <sup>g</sup>		5 × 10 <sup>3</sup>
hLT $\alpha$	L929			1 × 10 <sup>7</sup>
mLT $\alpha$	WEHI			1 × 10 <sup>5</sup>
hLT $\alpha$	WEHI			5 × 10 <sup>8</sup>

<sup>a</sup> 3 day growth assays with either L929 or WEHI 164 cells.

<sup>b</sup> Units are defined such that 100  $\mu$ l in the assay of a 1 U/ml solution will result in 50% cytotoxicity.

<sup>c</sup> Supernatants collected at 2.5 days post transfection and assayed directly. The mLT $\alpha$  was expressed either in pCDNA3 or pCDM8 with little difference. The hLT $\alpha$  was expressed in pCDM8.

<sup>d</sup> Estimated from Coomassie staining of an SDS-PAGE gel following immunoprecipitation with hTNF-R55-Ig.

<sup>e</sup> ELISA determination.

<sup>f</sup> Supernatant from insect cells was harvested 1 day postinfection and assayed directly.

<sup>g</sup> Protein concentration was determined by absorbance measurement assuming that 1 OD = 1 mg/ml.

interacted with the silica- and agarose-based column matrix. Similar sticking behavior was observed for *E. coli*-derived hLT $\alpha$  (23).

#### Comparison of the TNF-R55-mediated biological activities of human and murine LT $\alpha$

When insect cell-derived mLT $\alpha$  was assayed for activity in the standard 1-day cycloheximide L929 cytotoxicity assay or in a 3-day anti-proliferative version of this assay, no cytotoxic or growth inhibitory activity was observed. When the more sensitive WEHI 164 cells were tested, only modest levels of activity were observed, with the sp. act. being 1,000- to 10,000-fold less than the equivalent human LT $\alpha$  (Table I). To test whether mLT $\alpha$  was rendered inactive by the acid elution steps from the affinity column, the sp. act. of mLT $\alpha$  was measured directly in the insect cell supernatants without purification. The concentration of mLT $\alpha$  was determined with a specific ELISA for mLT $\alpha$ . This ELISA did not show cross-reactivity with mTNF and had a lower limit of sensitivity of about 10 ng/ml (Fig. 2). As shown in Table I, these culture supernatants also lacked the predicted biological activity. Since proteolysis can occur in baculovirus-infected insect cell supernatants, the amount of mLT $\alpha$  secreted was determined on days 1, 2, and 3 postinfection, and these values were compared with the bioactivity measurements. There was no indication that active material was made preferentially either early or late following infection, suggesting that subsequent cleavage events do not dramatically activate or inactivate the molecule. Lastly, human LT $\alpha$  is very active whether produced from bacteria, insect cells, or CHO cells, indicating that the extent or type of glycosylation does not dramatically affect the sp. act., and hence, altered glycosylation is unlikely to account for the poor activity of the mLT $\alpha$  (18, 24).

To determine whether mLT $\alpha$  was also inactive when secreted from a mammalian cell line, COS cells were transfected with all the mLT $\alpha$  constructs described in *Materials and Methods*. Similar

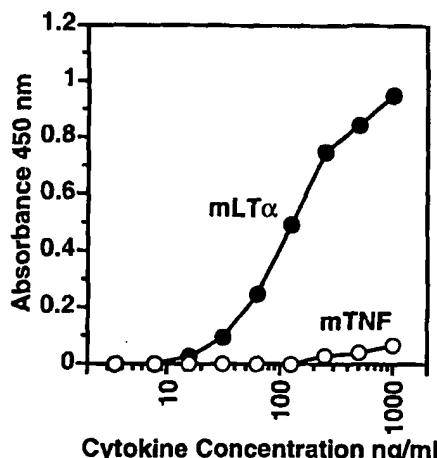


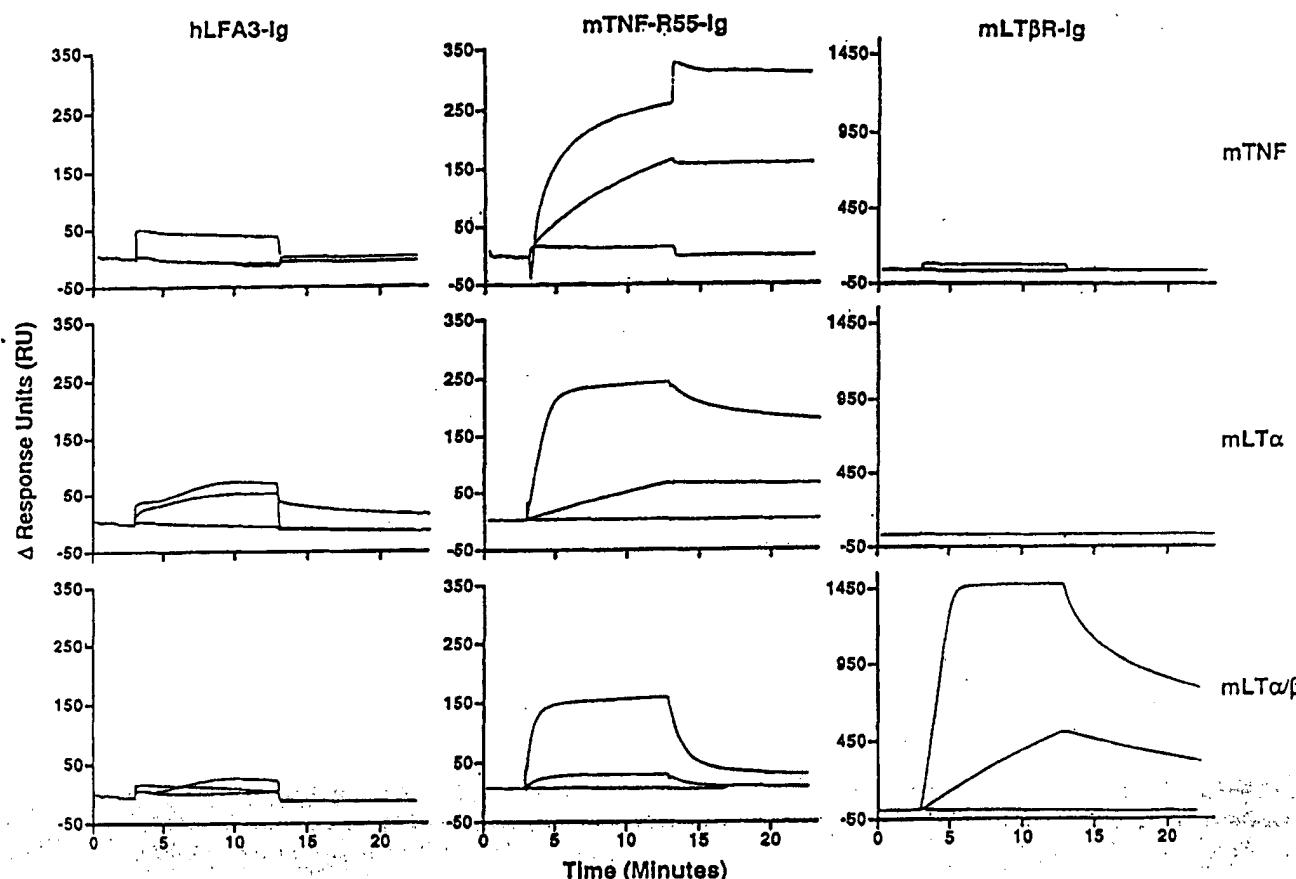
FIGURE 2. ELISA analysis showing the specificity of the assay for mLT $\alpha$  and the lack of cross-reactivity with mTNF.

constructs were prepared with the murine and human LT $\alpha$  cDNAs in terms of both the expression vector and the nature of the 3' untranslated regions. In two vectors (CH241 and 244), the 3' untranslated region was truncated to eliminate any regulatory elements. Immunoprecipitations of radiolabeled COS supernatants with soluble TNF-R55-Ig showed that both proteins were expressed well regardless of the presence of an optimal 5' Kozak element or 3' untranslated regions. ELISA quantitation showed the presence of 10 to 20 ng/ml of mLT $\alpha$  in the COS cell supernatants, yet no detectable WEHI 164 activity was found. By comparison, hLT $\alpha$  protein was expressed at similar levels in COS cells, yet the bioassays showed the expected high sp. act. The bioassays were run in either the 3-day anti-proliferative format or in a 24-h cytotoxicity assay in the presence of cycloheximide with no significant differences. These observations show that secreted mLT $\alpha$  has greatly reduced biological activity compared with its human counterpart in a mTNF-R55-based cytotoxicity assay.

#### Characterization of mLT $\alpha$ $\beta$ complexes

Human LT $\alpha$ 2 $\beta$ 1 and LT $\alpha$ 1 $\beta$ 2 complexes have been prepared and characterized previously (3). In a similar fashion, we have prepared murine versions of these forms (see Fig. 1). Coinfection of insect cells with both mLT $\alpha$ - and myc-mLT $\beta$ -encoding baculoviruses led to the expression of mLT $\alpha$  $\beta$  complexes in a manner similar to the human trimers. The insect cell supernatants were loaded directly onto a mLT $\beta$ -Ig column, and bound forms were eluted. This fraction was called the generic mLT $\alpha$  $\beta$  complex. SDS-PAGE and C<sub>4</sub> reverse phase HPLC analyses of this preparation showed that the generic mLT $\alpha$  $\beta$  complex is composed of approximately 60% mLT $\alpha$  and 40% mLT $\beta$ , suggesting that this preparation is a mixture of mLT $\alpha$ 1 $\beta$ 2 and mLT $\alpha$ 2 $\beta$ 1 forms (data not shown). There was no evidence of pure mLT $\alpha$  in this preparation by ion exchange chromatographic analysis (data not shown). Gel exclusion chromatography using a TSK G3000 column in the presence of 0.5 M NaCl showed that this preparation migrated at the size expected for a compact trimer (data not shown).

The purification of the two human trimers relied on the selective depletion of LT $\alpha$ 2 $\beta$ 1 from the supernatant by absorption onto an hTNF-R55-Ig affinity column. In the case of mLT $\alpha$  $\beta$ , this depletion was found to occur inefficiently. When generic mLT $\alpha$  $\beta$  was passed over an hTNF-R55-Ig column to deplete the mLT $\alpha$ 2 $\beta$ 1 form, the eluate from this column was primarily mLT $\alpha$ 2 $\beta$ 1. Gel exclusion chromatography showed the material to be mostly in a



**FIGURE 3.** BIACore analysis of the binding of mTNF, mLTA, and mLTA $\beta$  to hLFA-3-Ig- (control), mTNF-R55-Ig-, and mLTPR-Ig-coated chips, as indicated by the panel labels, as a function of time. Binding curves are shown using 0, 0.2, and 2  $\mu$ g/ml ligand concentrations. In the case of mLTA, which had some nonspecific binding to hLFA-3-Ig, the background on an hLFA-3-Ig-coated chip was subtracted from the mTNF-R55-Ig and LT $\beta$ R-Ig signals in the center and left panels. Chips were exposed to the ligand in the interval from 3 to 13 min. The lower level of ligand binding to the mTNF-R55-Ig-coated chips relative to binding to the mLTPR chip suggested that some receptor inactivation may have occurred during coupling.

trimeric form, as was observed for the human forms, and C<sub>4</sub> reverse phase HPLC confirmed the expected 2:1 ratio of LT $\alpha$  to LT $\beta$  (data not shown). Ion exchange chromatography also was consistent with relatively pure mLTA $\alpha$ 2 $\beta$ 1. The flow-through from the TNF-R55 affinity column should contain LT $\alpha$ 1 $\beta$ 2; however, HPLC analysis revealed a ratio closer to 1:1 of LT $\alpha$ :LT $\beta$  instead of the expected 1:2. The lack of purity is believed to result from a poor affinity of the LT $\alpha$ 2 $\beta$ 1 for the TNF-R55 affinity column, and therefore not all LT $\alpha$ 2 $\beta$ 1 is depleted, leading to an incorrect ratio (data not shown). Given our inability to generate pure mLTA $\alpha$ 1 $\beta$ 2, we have focused on the properties of generic mLTA $\alpha$ 2 $\beta$ 1 in the bioassays.

Analysis of the binding of LT $\alpha$ 2 $\beta$ 1 and LT $\alpha$ 1 $\beta$ 2 forms to LT $\beta$ R and TNF-R55 is complicated due to the heterogeneous nature of the binding clefts. With the human ligands, BIACore analysis has been informative in defining the cleft interactions, and these results will be described elsewhere (J. L. Browning, manuscript in preparation). In this study, we have used BIACore analysis in a qualitative fashion to ascertain whether the properties of the murine ligands were roughly comparable to those of the human ligands and secondly to determine whether the mLTA molecule bound more weakly to mTNF-R55 than did hLT $\alpha$ . Figure 3 shows Sensorgrams from a preliminary study of the binding of mTNF, mLTA, and mLTA $\beta$  to hLFA-3-Ig- (a control Ig fusion protein), mTNF-R55-, and mLTPR-Ig-coated chips. At 20  $\mu$ g/ml, mLTA

bound very well to the control hLFA-3-Ig-coated chip (binding to the 3000 resonance units level; data not shown), and this background was visible even at lower concentrations (Fig. 3). We have rarely observed such background binding or sticky behavior in studies of TNF family members. Notably, mutants of hLT $\alpha$  that have lost the ability to signal through the TNF-R, e.g., D50N and Y108F, also are sticky and behave like mLTA. The heteromeric LT $\alpha$ 2 $\beta$  ligands were not sticky. The comparison of mTNF and mLTA binding to mTNF-R55-Ig showed a slow on-rate for mTNF coupled with almost no perceptible off-rate, which would indicate relatively tight binding. Human TNF has a faster on-rate for binding to both murine and human TNF-R55-Ig chips (data not shown). Mouse LT $\alpha$  also bound to the mTNF-R55-Ig chip; however, dissociation events were now readily apparent upon removal of the ligand from the buffer. In the case of mTNF, saturated binding was already being approached at 0.2  $\mu$ g/ml, which was not observed at this concentration of mLTA. Thus, mLTA appears to differ from mTNF by having a lower  $K_d$  as well as a faster off-rate. Equilibrium Scatchard analysis using BIACore data indicated that the  $K_d$  for mLTA binding to the immobilized mTNF-R55-Ig chip was in the range of 50 to 200 nM, although this determination was rather crude due to the background binding problems (data not shown). Nonetheless, this binding was considerably weaker than the roughly 1 nM  $K_d$  typically described for hTNF or hLT $\alpha$  interactions with TNF-R55-Ig. These data suggest that the LT $\alpha$ :LT $\alpha$

cleft, i.e., the receptor binding surface that makes productive contacts between adjacent subunits in the crystal structure of hLT $\alpha$ :hTNF-R55, cannot bind as well in mLT $\alpha$ :mTNF-R55.

Mouse LT $\alpha\beta$  bound to the mTNF-R55-Ig-coated chip, but the off-rate was fast, indicating very weak binding. Similar binding properties were noted for hLT $\alpha$ 2 $\beta$ 1:hTNF-R55-Ig system. This relatively fast off-rate may account for the poor ability of a TNF-R55-Ig column to deplete conditioned medium of the LT $\alpha$ 2 $\beta$ 1 form. The generic mLT $\alpha\beta$  preparation interacted with the mLT $\beta$ R-Ig-coated chips in a manner consistent with higher affinity binding. In experiments in which generic mLT $\alpha\beta$  was bound to a TNF-R55-Ig affinity column and pure mLT $\alpha$ 2 $\beta$ 1 was eluted, this material dissociated from the mLT $\beta$ R-Ig chip more quickly than the mLT $\alpha\beta$  preparation, which mimics the behavior of pure hLT $\alpha$ 2 $\beta$ 1 ligand (data not shown). Conversely, the TNF-R55-Ig flow-through is presumably enriched for mLT $\alpha$ 1 $\beta$ 2 and displayed a slower off-rate than the mLT $\beta$ R-Ig chip, which is also consistent with the behavior of hLT $\alpha$ 1 $\beta$ 2. Thus, while we have not succeeded in purifying mLT $\alpha$ 1 $\beta$ 2 to homogeneity, the experiments described here suggest that the mLT $\alpha\beta$  ligands behave like their human counterparts and the purification difficulty stems from the intrinsically poor affinity of mLT $\alpha$ 2 $\beta$ 1 for TNF-R55.

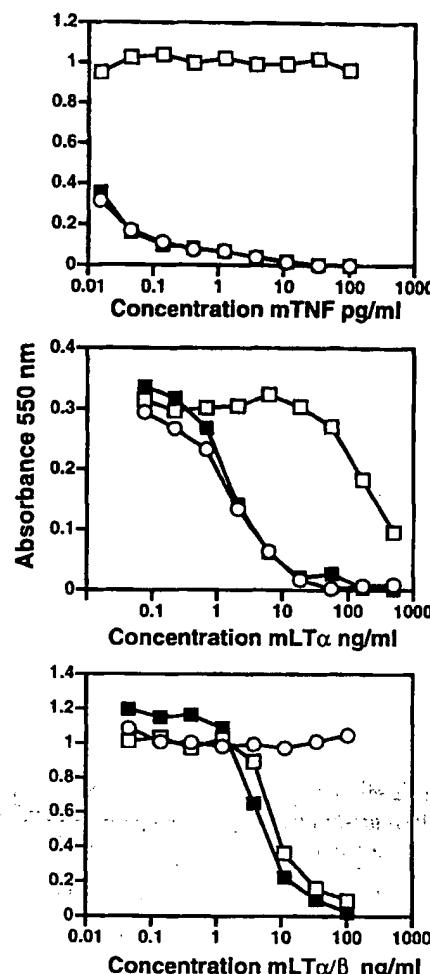
#### Characterization of anti-mLT $\beta$ R mAbs

To differentiate between LT $\beta$ R- and TNF-R-mediated activities of the various secreted mouse LT forms, we required 1) Abs that would allow the delineation of receptor-positive cell types, and 2) Abs with antagonistic properties. Such a panel of anti-LT $\beta$ R mAbs has been well characterized in the human receptor system (16), and similar methods were used in this study. Six hamster anti-mLT $\beta$ R mAbs were developed based on direct ELISA screening, the ability to block mLT $\beta$ R-Ig binding to mLT $\alpha\beta$  in an ELISA format, the ability to stain WEHI 164 and L929 cells in a FACS assay, and the ability to block the adhesion of surface LT-positive II-23 cells onto mLT $\beta$ R-coated plates. BIACore epitope mapping was also conducted to allow some preliminary grouping of the various mAbs. All the mAbs except AFB1 appeared to bind to closely spaced epitopes on the receptor, as determined by BIACore epitope mapping. Three of the mAbs, ADB10, ACH6, and AFH6, effectively blocked ligand receptor interactions, as assessed using either the ELISA binding or cell adhesion formats (data not shown), although ADB10 was weaker in these tests. All the mAbs were able to detect receptor on the surface of WEHI 164 or RAW 264.7 cells (data not shown).

#### Comparison of the biological activities of the mLT $\alpha$ and mLT $\alpha\beta$ forms

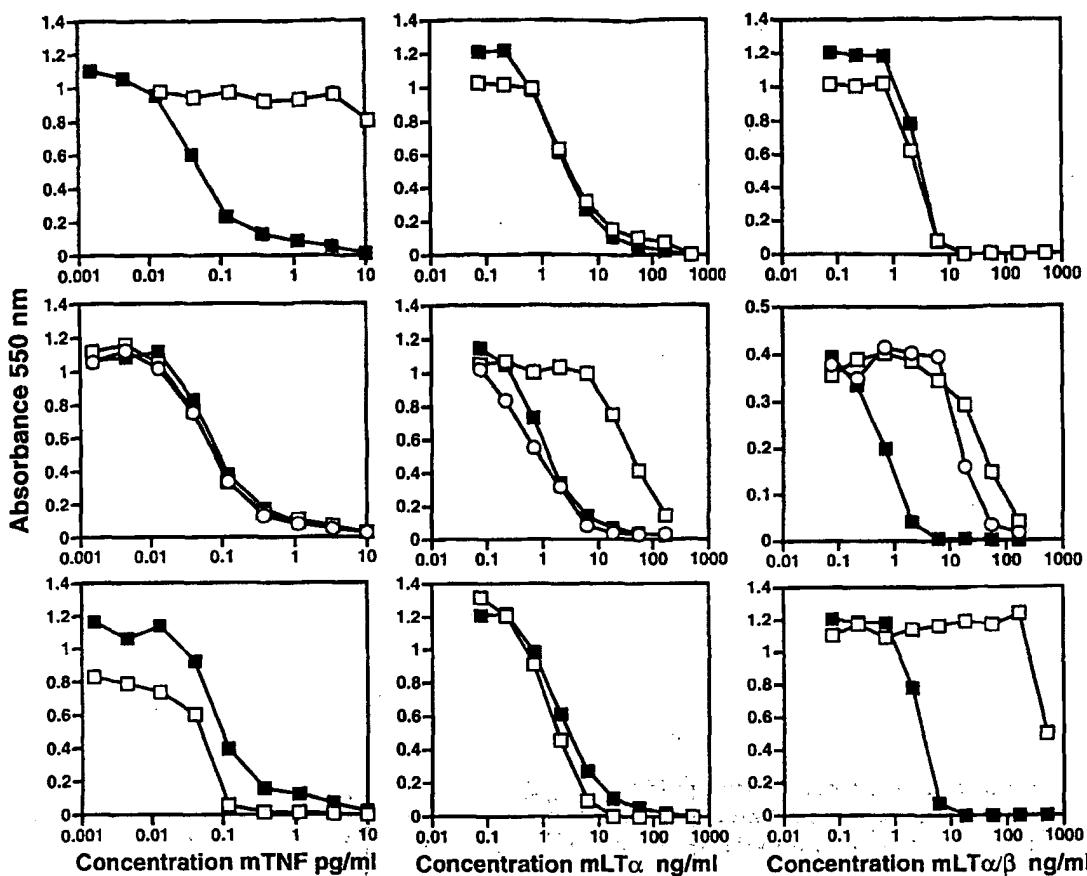
Using the sensitive WEHI 164 assay, we compared the cytotoxic activities of mLT $\alpha$  and the generic mLT $\alpha\beta$  eluate from the LT $\beta$ R-Ig affinity column. Both LT forms had cytotoxic activity on these cells, although neither compound was as potent as either mouse or human TNF or human LT $\alpha$ . To determine whether there was mLT $\alpha$  contamination in the mLT $\alpha\beta$  preparation, we added either mTNF-R55-Ig or mLT $\beta$ R-Ig to the assays (Fig. 4). As in the human case (16), soluble TNF-R55-Ig inhibited mLT $\alpha$  activity, whereas only mLT $\beta$ R-Ig blocked mLT $\alpha\beta$  activity. This experiment indicated that there is little mLT $\alpha$  homotrimer present in the mLT $\alpha\beta$  preparations. LT $\alpha$ 2 $\beta$ 1 can potentially cross-link both TNF-R55 and mLT $\beta$ R into a common complex; however, the lack of a TNF-R55-Ig-inhibitable component in the mLT $\alpha\beta$  preparation suggests that such a mechanism was not effective in the WEHI 164 assay.

Human HT-29 cells were killed by activation of the LT $\beta$ R with either agonistic mAbs or soluble LT $\alpha$ 1 $\beta$ 2 ligand (16), and it is



**FIGURE 4.** Activity analysis of recombinant (r-) mTNF, r-mLT $\alpha$ , and r-mLT $\alpha\beta$  using a 3-day WEHI-164 proliferation assay. Cells were grown in the presence of the indicated amount of cytokine (■) with either 10  $\mu$ g/ml of soluble mTNF-R55-Ig (□) or mLT $\beta$ R-Ig (○).

believed that the killing of WEHI 164 cells by mLT $\alpha\beta$  represents a similar LT $\beta$ R-mediated signaling event. The various anti-mTNF, mLT $\alpha$ , mLT $\beta$ , and mLT $\beta$ R mAbs were added to the WEHI 164 bioassay both to assess whether engaging the LT $\beta$ R could directly trigger cell death and to determine the specificities of the various Abs for their respective ligands. Figure 5 shows the results of such an analysis, and Table II summarizes all the results. The hamster anti-mTNF Ab TN3.19.12 that reportedly cross-reacted with both TNF and LT $\alpha$  could only neutralize mTNF and not mLT $\alpha$  or mLT $\alpha\beta$ ; thus, it is specific for TNF. Similar results were obtained using the rat anti-mTNF mAb MP6-XT22. In contrast, the anti-mLT $\alpha$  mAb AFB3 can block both mLT $\alpha$ - and mLT $\alpha\beta$ -induced cell death, but has no effect on TNF activity. The anti-mLT $\beta$  mAb BBF6 was only able to inhibit the activity of mLT $\alpha\beta$ , as expected. The ability of both anti-mLT $\alpha$  mAbs and the goat anti-mLT $\alpha$  serum to block mLT $\alpha\beta$  activity was expected, but curiously was not observed in the human system (16). The reasons for the lack of inhibition by anti-hLT $\alpha$  mAbs are not clear, but possibly a different set of LT $\alpha$  epitopes is recognized by the hamster or goat repertoires. Lastly, an anti-mLT $\beta$ R mAb, ACH6, blocked only the activity of mLT $\alpha\beta$  and not that of mLT $\alpha$  or TNF, proving that the mLT $\beta$ R is involved in the death events induced by mLT $\alpha\beta$ . The potentiation of the activity of mTNF by ACH6 is believed to result



**FIGURE 5.** Analysis of the abilities of various mAbs to inhibit mTNF, mLT $\alpha$ , or mLT $\alpha$ / $\beta$  antiproliferative activity in a 3-day WEHI 164 assay. In the top three panels cytokines were premixed with (□) or without (■) 10  $\mu$ g/ml of rat anti-mTNF; in the middle three panels they were premixed with 10  $\mu$ g/ml of control hamster IgG (■), anti-mLT $\alpha$  mAb AFB3 (□), or anti-mLT $\beta$  mAb BBF6 (○); and in the bottom three panels they were premixed with no added mAbs (■) or with an anti-mLT $\beta$ R mAb ACH6 (□). Some experiments were performed on different days, accounting for the variable maximal OD values.

**Table II.** Ability of Abs to mTNF, mLT $\alpha$ , mLT $\beta$ , and receptors to inhibit the cytotoxic effects of TNF and LT forms on WEHI 164 cells

Antibody	Type	Inhibition of Cytotoxicity <sup>a</sup>		
		mTNF	mLT $\alpha$	mLT $\alpha$ / $\beta$
TN3-19.12	hamster anti-mTNF	+	—	—
MP6-XT22	rat anti-mTNF	++	—	—
Pre-mLT $\alpha$ No. 3	goat prebleed	—	—	—
Anti-mLT $\alpha$ No. 3	goat anti-mLT $\alpha$	—	+++	+++
AFB3	hamster anti-mLT $\alpha$	—	++	++
AF6	hamster anti-mLT $\alpha$	—	+/-	+
BBF6	hamster anti-mLT $\beta$	—	—	++
CMB1	hamster anti-mLT $\beta$	—	—	++
AFB1	hamster anti-mLT $\beta$ R	ND	ND	(-) <sup>b</sup>
ACH6	hamster anti-mLT $\beta$ R	—	—	+++
AFH6	hamster anti-mLT $\beta$ R	ND	ND	+++
ADB10	hamster anti-mLT $\beta$ R	ND	ND	+
CCG6	hamster anti-mLT $\beta$ R	ND	ND	—
CEF8	hamster anti-mLT $\beta$ R	ND	ND	—
TR75-32	hamster anti-mTNF-R75	+	—	—

<sup>a</sup> WEHI 164 3-day antiproliferative assay. In this grading system, the number of plus signs indicates approximately the log base 10 shift in the dose response curves.

<sup>b</sup> This mAb augmented mLT $\alpha$ / $\beta$  activity.

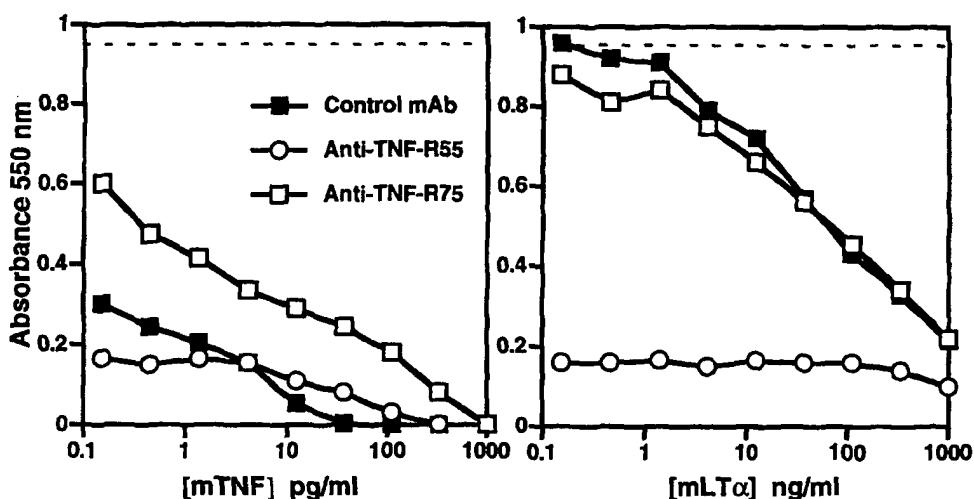
from some synergistic interplay between the signal transduction components used by both the TNF-R and LT $\beta$ R. Such potentiation was observed in the human system (16). In other assays both in

vitro and in vivo, the anti-mLT $\beta$ R mAb ACH6 acted as an agonist, and we suspect that the antagonistic activity of this mAb shown here may be more reflective of effects on this possibly predominantly LT $\alpha$ 2 $\beta$ 1-containing preparation. Mixed antagonistic/agonistic activity of anti-receptor mAbs is a common observation in this field.

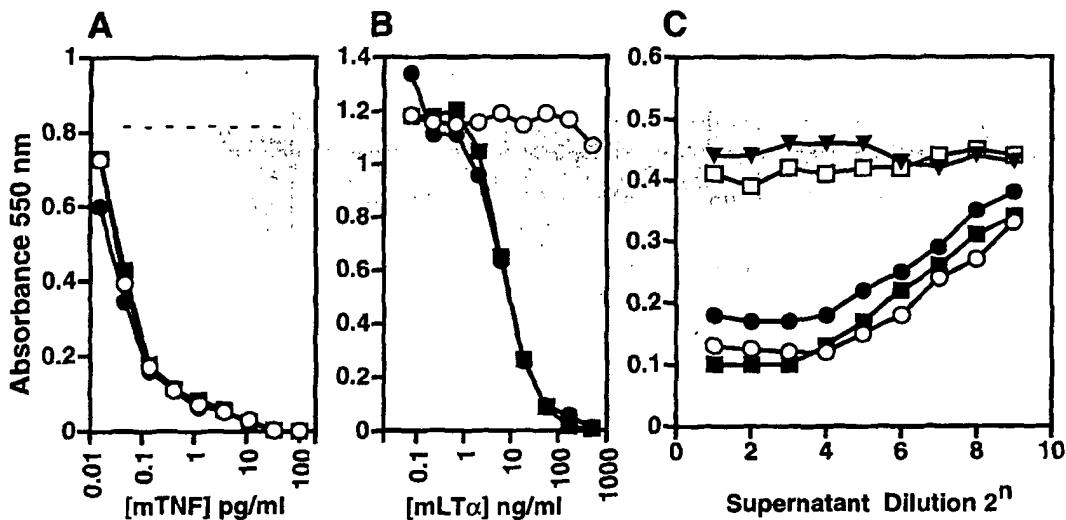
To determine which TNF-R was responsible for mTNF and mLT $\alpha$  signaling in WEHI 164 cells, anti-mTNF-R55 and anti-mTNF-R75 mAbs were added to the WEHI 164 assay. Figure 6 shows that inhibition of mTNF-R75 shifted the mTNF dose-response curve 10-fold, whereas the mLT $\alpha$  curve was not affected. The Ab, 55R-170, which is reportedly an antagonistic mTNF-R55 mAb in the L cell-based TNF assay, was a potent agonist mAb in the WEHI 164 system (Fig. 6). With human cells, hTNF-R75 is often a component of hTNF cytotoxicity in many systems, yet with F54 fibroblasts, anti-hTNF-R75 blocked only TNF and not LT $\alpha$  activity (25). In fact, differences between LT $\alpha$  and TNF in some systems appear to stem from the inability of LT $\alpha$  to interact with TNF-R75 (26). Thus, the lack of a TNF-R75 component to mLT $\alpha$  signaling in WEHI cells indicates that mouse and human LT $\alpha$  are qualitatively similar, yet mLT $\alpha$  has a much lower sp. act.

#### Analysis of cytotoxic activity secreted by activated lymphocytes

As the ELISA assay with its lower limit of about 10 ng/ml may not have sufficient sensitivity to detect mLT $\alpha$  in actual cell supernatants, we relied on a TNF-R55-based cytotoxicity assay to detect



**FIGURE 6.** Use of an anti-mTNF-R75 mAb to define the contribution of this receptor to mTNF and mLT $\alpha$  signaling in WEHI 164 cells. Cells were preincubated for 30 min with 10  $\mu$ g/ml of control hamster mAb Ha4/8 (■), anti-mTNF-R75 (□), or anti-mTNF-R55 (○) and then diluted 1/1 into ligand-containing medium. Growth was measured after 3 days. Control growth is indicated by the dotted line, and this OD was unaffected by the control Ha4/8 or anti-TNF-R75 mAbs, but was reduced to 0.16 OD by the anti-mTNF-R55 mAb in the absence of any ligand.



**FIGURE 7.** Use of selective anti-mTNF and mLT $\alpha$  Abs to define the contributions of these two cytokines to the cytotoxic activity released from activated mouse spleen cells. A and B show the ability of an anti-mLT $\alpha$  goat serum to block the antiproliferative activity of recombinant mLT $\alpha$  (B), but not that of mTNF (A). Compared are the effects of various concentrations of cytokine in the presence of no addition (■), a 1/100 dilution of either preimmune goat serum (●) or immune goat serum (○) in a 3-day WEHI 164 cytotoxicity assay. C shows the cytotoxic activity of conditioned medium from mouse spleen cells cultured for 1 day with 1  $\mu$ g/ml staphylococcal enterotoxin B. Assays were conducted with no additions (■) or with 10  $\mu$ g/ml rat anti-mTNF mAb MP6-XT22 (□), a 1/100 dilution of preimmune goat serum (●), a 1/100 dilution of goat anti-mLT $\alpha$  serum (○), or a combination of rat anti-mTNF mAb with the anti-mLT $\alpha$  goat serum (▼). Data are from a 1-day WEHI 164 cytotoxicity assay in the presence of cycloheximide.

potential cytokine. To ensure that we would be able to effectively block natural mLT $\alpha$ , we prepared a polyclonal antiserum to mLT $\alpha$  to complement the hamster anti-mLT $\alpha$  mAbs. A goat polyclonal antiserum to mLT $\alpha$  reacted well with mLT $\alpha$  and not mTNF in both ELISA and Western formats and weakly bound to hLT $\alpha$  in an ELISA (data not shown). When added to a mLT $\alpha$  WEHI 164 cytotoxicity assay, the goat antiserum effectively blocked the mLT $\alpha$  activity, yet had no effect on mTNF activity (Fig. 7, A and B). This serum was only able to partially block the activity of hLT $\alpha$  with a 1/100 dilution of the serum, shifting the dose-response curve by a factor of 3 to 5 (data not shown).

A number of human lymphocyte tumor lines were identified that could readily secrete hLT $\alpha$  following PMA activation. We examined two murine cell lines, TIMI.4 and CH1, that had been shown to express both mLT $\alpha$  and mLT $\beta$  and display surface LT complexes after PMA treatment (17a). The supernatants were assayed for any residual cytotoxic activity that would not be blocked by the rat anti-TNF mAb MP6-XT22, and we found that all detectable activity could be accounted for by TNF secretion. To further address this question, mouse splenic lymphocytes were activated by various methods, and the supernatants were analyzed. Table III summarizes these results, and again there was no evidence of any

Table III. Secretion of TNF and LT $\alpha$  by activated mouse spleen cells

Treatment	mIL-2 <sup>b</sup> (ng/ml)	WEHI 164 Cytotoxic Activity in Supernatant (U/ml)				
		None	Rat-anti-mTNF	Added Blocking Agent <sup>a</sup>		Anti-mTNF + anti-mLT $\alpha$
				Pre	Post	
Control	0.09	4	<1	ND	ND	ND
SEB	11	147	<1	97	256	<1
SEB + PMA	7	64	<1	21	48	<1
Anti-CD3 + IL-2	31	74	<1	56	111	<1

<sup>a</sup> One-day WEHI 164 cytotoxicity assay with 10  $\mu$ g/ml cycloheximide. Units represent the reciprocal of the dilution of the supernatant needed to obtain 50% killing.

<sup>b</sup> ELISA determination.

Rat anti-mTNF Ab was included at 10  $\mu$ g/ml; goat polyclonal anti-mLT $\alpha$  was included at a 1:200 dilution.

non-TNF component to the cytotoxic activity. Figure 7C illustrates one example from Table III in which >99.5% of the activity was blocked by anti-mTNF, and the addition of the anti-mLT $\alpha$  anti-serum to the anti-mTNF-blocked solution did not result in a further decrease in activity. Therefore, in the activated spleen cell system, if secreted mLT $\alpha$  was present it accounted for <0.5% of the total cytotoxic activity in these analyses. Moreover, based on these data, if mLT $\alpha$  of a sp. act. comparable to that of mTNF or hLT $\alpha$  was secreted from these cells, the concentration of active material would be <1 pg/ml. Spleen cell supernatants were examined 48 h after activation, and lower levels of cytotoxic activity were observed, again with mTNF accounting for all the activity. Since the 1-day cytotoxicity assay in the presence of cycloheximide and the 3-day antiproliferative assays may actually measure different events as has been postulated for TNF (27), the above experiments were repeated with the 3-day assay, and qualitatively identical results were obtained.

## Discussion

The issue of the relative functions of the LT and TNF systems has been debated for some time. Conventional views have centered on the idea that secreted TNF and LT have similar actions, a view based largely on the properties of the human ligands. Recent studies have shown that a separate LT signaling pathway exists by which the surface heteromeric complex binds to the LT $\beta$ R (5). Thus, the question remains unresolved whether a biologically relevant mLT $\alpha$ :TNF-R55 interaction occurs. As described in this paper and the accompanying work, we have produced recombinant forms of murine LT $\alpha$  and LT $\alpha$  $\beta$  complexes as well as Abs to mLT $\alpha$ , mLT $\beta$ , and mLT $\beta$ R and defined their properties. This analysis leads to three major conclusions: 1) recombinant mLT $\alpha$  can only poorly induce mTNF-R55-mediated cytotoxicity; 2) primary splenic lymphocytes do not secrete substantial amounts of a mLT $\alpha$  form with high sp. act., and 3) the heteromeric mLT $\alpha$  $\beta$  ligand can signal cell death directly via activation of the mLT $\beta$ R.

The LT field has been severely hampered by the basic lack of any biochemical knowledge of murine LT forms. The fact that in the roughly 9 yr since cloning of the murine homologue of LT $\alpha$  there has not been a single publication describing the biochemical properties of this cytokine suggests that previous attempts have encountered unexpected difficulties. In this work, we have found that recombinant mLT $\alpha$  is not difficult to express; however, it does not have appreciable activity in the conventional TNF-R55-based

L929 or WEHI 164 cytotoxicity assays. In our original hypothesis, the surface LT $\alpha$  $\beta$  heteromeric complex represented the core component of the LT system, and we entertained the notion that the TNF-like properties of hLT $\alpha$  were an epiphenomenon. Furthermore, we speculated that mLT $\alpha$  may not share the TNF-like signaling properties of hLT $\alpha$ . Because the lack of the expected activity in a recombinant molecule demanded a careful analysis of possible technical difficulties, the molecule was expressed in two different eukaryotic systems, i.e., transiently in the monkey COS cell line and in insect cells. Both systems readily generated human and mouse LT proteins; however, only hLT $\alpha$  was obtained in a highly active form. Both proteins lack cysteine residues in the extracellular domain, indicating that proper disulfide bonding was not a causal factor. Multiple independent cDNA clones as well as genomic sequence were found to be identical, eliminating propagation of one mutated cDNA sequence as a possibility. N-terminal sequence analysis showed that mLT $\alpha$  was cleaved in the exact same position as hLT $\alpha$ , indicating that N-terminal proteolysis was also not a problem. Other proteolytic events are possible, and we carefully analyzed sp. act. at various times during the time course of insect cell expression when proteolysis does occur and looked either for early or late increases in sp. act. Such changes would point toward either inactivating or activating cleavage events, yet no indication of a critical proteolytic event was observed.

Not content with the analysis of recombinant mLT $\alpha$ , we questioned whether primary splenic lymphocytes could produce soluble LT $\alpha$  with high sp. act. It was known that in murine T cells, activation can lead to mLT $\alpha$  mRNA expression (6). Moreover, in the human system, activation of PBL led to the production of a mixture of both TNF and LT $\alpha$ , where LT $\alpha$  comprised up to 20% of the total cytotoxic activity (28–30). Based on these data, we expected that the supernatants of activated mouse spleen cells should also contain some LT $\alpha$ -based cytotoxic activity. To be sure that we could adequately discriminate between the natural TNF and LT $\alpha$  forms, we relied not only on a specific TNF mAb to block TNF, but also on a goat anti-serum to mLT $\alpha$  that had excellent neutralizing properties. The spleen cell supernatants were cytotoxic to the very sensitive WEHI 164 cells, and the activity was completely blocked by an anti-TNF mAb. The accuracy of these measurements was such that a 1% mLT $\alpha$  component should have been detected easily. Thus, at least by comparison with the behavior of human PBLs, mLT $\alpha$  capable of inducing TNF-R55-mediated cytotoxicity was not secreted by activated mouse splenic lymphocytes. These experimental results also agree with a previous analysis of such cytotoxic activity using a polyclonal anti-mTNF anti-serum (11) and the TNF-specific TN3-19.12 mAb (see below) (21). While we cannot find a high sp. act. mLT $\alpha$  form secreted from activated spleen cells, it is possible that other cell sources, such as mouse PBLs, may express such material under the right conditions and thus would mimic more closely the results found with human cells.

The Ab analyses presented here and in the accompanying work show that cross-reactivity between TNF and LT in the murine system does not occur. The previous report that the hamster anti-mTNF mAb TN3 neutralized both mTNF and mLT $\alpha$  activity was based on the assumption that mLT $\alpha$  had TNF-R55-directed biological activity and that this activity was present in supernatants from activated lymphocytes (21). The direct measurement of mLT $\alpha$  activity showed that this mAb could only block mTNF activity and not mLT $\alpha$ . Therefore, the ability of this mAb to inhibit rodent models of experimental allergic encephalitis and diabetes results from the inhibition of mTNF and not from a combination of mTNF and mLT $\alpha$  (31, 32).

The secreted human and mouse LT $\alpha$  forms are 78% identical; therefore, why can only one form activate TNF-R55 efficiently? BIAcore analysis of the mL $\alpha$ :TNF-R55 interaction indicated that the molecule was relatively sticky, a property we have observed in the D50N and Y108F mutants of hLT $\alpha$ , and that these mutant LT $\alpha$  forms also cannot activate the hTNF-R55. Moreover, the preliminary BIAcore comparison of hLT $\alpha$  and mL $\alpha$  binding to mTNF-R55 indicated a lower affinity for the mL $\alpha$  interaction and a faster off-rate. The properties of the LT $\alpha$  forms should be mimicked by the LT $\alpha$ 2 $\beta$ 1 molecule, as it possesses an LT $\alpha$ :LT $\alpha$  cleft capable of binding TNF-R55 (3). As the LT $\alpha$ 2 $\beta$ 1 forms are not sticky in a BIAcore analysis, we examined the changes in the properties of hLT $\alpha$ 2 $\beta$ 1 upon introduction of the D50N mutation in the LT $\alpha$  component (data not shown). This mutation lowers the binding affinity of the LT $\alpha$ :LT $\alpha$  cleft of hLT $\alpha$ 2 $\beta$ 1 for TNF-R55 by about three- to fivefold. Extrapolating to the LT $\alpha$  trimer, it would appear that a small decrease in the binding affinity could be sufficient to essentially eliminate productive signaling and cytotoxicity. Therefore, small changes in the mL $\alpha$  molecule could have dramatic biological consequences.

What are the biochemical differences that could lead to poor performance? Based on gel exclusion chromatography, the mouse version was predominantly a trimer, as has been well documented in the human case (2). One possibility for the poor ability to activate TNF-R55 may lie in a lack of perfect packing or even imperfect 3-fold symmetry of the subunits in the mL $\alpha$  trimer. It is reasonable to assume that the LT $\alpha$  and LT $\beta$  chains evolved in a manner that optimized heteromeric assembly, and it is possible that the optimal structure for heteromerization is not correct for homotrimer packing. For example, hLT $\beta$  in the absence of hLT $\alpha$  does not appear to homotrimerize effectively (3). A less drastic manifestation may simply be imperfect receptor binding sites in the resultant trimer. This explanation would suggest that the difference between the murine and human LT systems lies in the subtleties of the evolutionary paths following the gene duplication events that led to their existence. In this context, it is useful to speculate on how the LT system originated. Gene duplication events probably created the current three-gene TNF cluster. Immediately following one duplication event, there would exist two very similar gene products that not only bound to the same receptor, but could "homo"-trimerize. Typically, one gene product would be expected to undergo mutations that would initiate a new pathway, and that gene would now drift in a direction in which oligomerization with the other related gene product is no longer possible. A likely scenario that can explain the LT system is that the initial drift of one Ur-LT member led to preferential assembly with the other Ur-LT. If a receptor developed that recognized this heteromer, then subsequent evolution of the pair would proceed in lock step to reinforce the structures driving heteromerization. In the human case, LT $\alpha$  still can efficiently homotrimerize and has not lost its ability to signal via the TNF-R despite considerable drift in the amino acid sequence from TNF and LT $\beta$  (or vice versa). In contrast, it is possible that mL $\alpha$  has not retained this ability, hence the current state. A cowpox strain was found to encode a presumably defensive soluble TNF-R form that could inhibit TNF, but not LT $\alpha$ , activity, which could be consistent with the functional divergence of the TNF and LT pathways (33). A possible manifestation of this model may lie in a comparison between the murine and human LT $\alpha$  amino acid sequences. The solution of the crystal structure of the hLT $\alpha$ :TNF-R55 complex delineated contact regions between the ligand and the receptor (34). Most of these contacts are conserved in the mouse ligand; however, one receptor contact region (amino acids 155 through 159 of the Banner et al. (34) numbering) is substantially different in the

mouse ligand. Moreover, a V158D mutant of hLT $\alpha$  results in 100-fold reduced activity, and in this context the mouse ligand is a V158H mutagenized hLT $\alpha$ . Thus, this difference alone may explain the poor activity. This study was strictly limited to an analysis of cytotoxic activity. Even though mL $\alpha$  is unable to deliver an effective death signal via mTNF-R55, it is possible that other events, such as nuclear factor- $\kappa$ B activation, that use different signal transduction machinery may still occur and may be physiologically relevant. On the other hand, death is a very efficient readout of mTNF-R55 activation in this cell, requiring very few occupied receptors, and other signal transduction pathways will at best equal this efficiency. Mouse LT $\alpha$  could signal through other undefined receptors, and the genetic dissection of the LT pathway suggests that another LT receptor system may exist (see below).

The relative capabilities of human LT $\alpha$  and TNF to signal in various systems has vexed workers in this field for some time. Human LT $\alpha$  can be more effective than TNF in influencing the growth of fibroblasts and EBV-transformed B cells, and these effects in long term cultures may stem from the better stability of LT $\alpha$  (35, 36). On the other hand, hLT $\alpha$  is less potent than hTNF in many systems despite similar affinities for the TNF-R55 (25, 35). It appears that the presence of TNF-R75 improves the signaling of hTNF, but not that of hLT $\alpha$ , which is consistent with the results presented here. Exactly why hLT $\alpha$ 's effects vary relative to TNF remains unresolved (25). It is possible that relatively subtle changes in off-rates could shorten the productive lifetime of a receptor-ligand complex, rendering it incapable of assembling the requisite intracellular signal transduction components, and these differences may underlie the lack of activity in the mL $\alpha$  case. Such off-rate effects could be compensated by faster on-rates and hence may not be manifested in analyses of the equilibrium binding constants.

Coexpression of the mL $\alpha$  and mL $\beta$  molecules led to their assembly into heteromeric trimers, basically as described for the human counterparts. The engineered soluble form of hLT $\alpha$ 1 $\beta$ 2 binds to the LT $\beta$ R with high affinity, can signal the death of some cell lines (16), activates nuclear factor- $\kappa$ B, and can induce HIV expression in the U1 monocyte line (37), (W. L. Marshall et al., manuscript in preparation), yet lacks potent proinflammatory activity (15). Here we have shown that mL $\alpha$ 1 $\beta$ 2 can induce the death of the WEHI 164 cell line in an LT $\beta$ R-specific manner. The potency of generic mL $\alpha$ 1 $\beta$ 2 is roughly equal to that of the mL $\alpha$  ligand, and as such is vastly weaker than that of mTNF, hTNF, or hLT $\alpha$ . It is currently questionable how well a soluble ligand can mimic the activity of a normally membrane-bound form, and this area requires more study. Despite the relatively poor sp. act. of the soluble mL $\alpha$ 1 $\beta$ 2 preparation, this cytotoxicity assay shows that a LT $\beta$ R signaling pathway can induce cell death in a murine system. As was observed in the human system, TNF-R55-Ig does not inhibit the mL $\alpha$ 1 $\beta$ 2-mediated killing event (3). Thus, the mouse LT $\alpha$ 1 $\beta$ 2 system appears to closely resemble the human one, and in both cases, a TNF family receptor lacking a canonical death domain can induce cell death.

Given this background, it is of interest to consider this model in light of the results of the historical data and recent genetic disruption of the LT and TNF systems. Genetic disruption of the LT $\alpha$  gene in mice led to a loss of all lymph nodes and disorganization of the spleen (38–40). Other than the loss of Peyer's patches in the TNF-R55 knockout mice (41), these phenotypes have not been observed in either TNF-R- or TNF-deficient mice, indicating unique LT $\alpha$ -mediated activity that does not result from TNF mimicry (42). The emergence of the LT $\beta$  gene and the discovery of a

unique  $LT\beta R$ -mediated pathway suggested that the phenotype revealed by  $LT\alpha$  disruption could be attributed to the  $LT\beta R$  signaling pathway. In this scenario, the genetic disruption of the  $LT\alpha$ ,  $LT\beta$ , and  $LT\beta R$  genes should lead to the same phenotype. Treatment of pregnant mice with the  $LT\beta R$ -Ig fusion protein allowed its transport into the fetus, where development of the lymph nodes was inhibited (43). As this receptor can only bind to the  $LT\alpha\beta$  complex and not to  $LT\alpha$  alone, events that are blocked by this maternal delivery technique are definitely mediated by the  $LT\alpha\beta$  complex. Maternal treatment led to splenic disorganization, as was observed in the  $LT\alpha$  knockout mice, and the loss of peripheral lymph nodes and Peyer's patches, but not mesenteric lymph nodes. The phenotype of mice possessing a disrupted  $LT\beta$  gene closely resembles that of mice generated following maternal  $LT\beta R$ -Ig treatment (14, 44). As the mesenteric lymph nodes appear to be ablated in  $LT\alpha$  null mice, but not in  $LT\beta$  or  $TNF-R$  knockout mice,  $LT\alpha$  may be signaling through an as yet undefined receptor system. The issue of whether  $mLT\alpha$  can signal through  $TNF-R55$  has not been definitively clarified by the genetic manipulations, and one could interpret the lack of Peyer's patch development in the  $TNF-R55$  null mice as a result of interrupted  $mLT\alpha$ : $TNF-R55$  communication. Such an interpretation is also consistent with the presence of Peyer's patches in  $TNF$  null mice (42). The signaling pathway mediating this activity remains undefined, yet the  $mLT\alpha$  biochemical and activity data presented here suggest that  $mLT\alpha$  by itself does not signal via  $mTNF-R55$ .

**Note added in proof.** The  $TNF-R55$  knockout mice have been shown to possess Peyer's patches, albeit poorly filled ones (M. Pasparakis, L. Alexopoulou, M. Grell, K. Pfizenmaier, H. Bluthmann, and G. Kollrias. 1997. *Proc. Natl. Acad. Sci. USA* 94:6319). The presence of these nodes negates the premise that  $LT\alpha$  signals through  $TNF-R55$  to initiate Peyer's patch development.

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## References

- Browning, J. L., A. Ngam-ek, P. Lawton, J. DeMarinis, R. Tizard, E. P. Chow, C. Hession, G. B. O'Brine, S. F. Foley, and C. F. Ware. 1993. Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell* 72:847.
- Ware, C. F., T. L. VanArsdale, P. D. Crowe, and J. L. Browning. 1995. The ligands and receptors of the lymphotoxin system. *Curr. Top. Microbiol. Immunol.* 198:175.
- Browning, J. L., K. Miatkowski, D. A. Griffiths, P. R. Bourdon, C. Hession, C. M. Ambrose, and W. Meier. 1996. Preparation and characterization of soluble recombinant heteromeric complexes of human lymphotoxins alpha and beta. *J. Biol. Chem.* 271:8618.
- Aggarwal, B. B., and K. Natarajan. 1996. Tumor necrosis factors: developments during the last decade. *Eur. Cytokine Netw.* 7:93.
- Crowe, P. D., T. L. VanArsdale, B. N. Walter, C. F. Ware, C. Hession, B. Ehrenfels, J. L. Browning, W. S. Din, R. G. Goodwin, and C. A. Smith. 1994. A lymphotoxin-beta-specific receptor. *Science* 264:707.
- Millet, I., and N. H. Ruddle. 1994. Differential regulation of lymphotoxin (LT), lymphotoxin- $\beta$  (LT- $\beta$ ), and TNF- $\alpha$  in murine T cell clones activated through the TCR. *J. Immunol.* 152:4336.
- Pokholok, D. K., I. G. Maroulakou, D. V. Kuprash, M. B. Alimzhanov, S. V. Kozlov, T. I. Novobrantseva, R. L. Turetskaya, J. E. Green, and S. A. Nedospasov. 1995. Cloning and expression analysis of the murine lymphotoxin beta gene. *Proc. Natl. Acad. Sci. USA* 92:674.
- Lawton, P., J. Nelson, R. Tizard, and J. L. Browning. 1995. Characterization of the mouse lymphotoxin- $\beta$  gene. *J. Immunol.* 154:239.
- Fashena, S. J., W. L. Tang, T. Sarr, and N. H. Ruddle. 1990. The murine lymphotoxin gene promoter: characterization and negative regulation. *J. Immunol.* 145:177.
- Kuprash, D. V., O. A. Osipovich, D. K. Pokholok, M. B. Alimzhanov, A. Biragyn, R. L. Turetskaya, and S. A. Nedospasov. 1996. Functional analysis of the lymphotoxin- $\beta$  promoter: sequence requirements for PMA activation. *J. Immunol.* 156:2465.
- Laskov, R., G. Lancz, N. H. Ruddle, K. M. McGrath, S. Specter, T. Klein, J. Y. Djeu, and H. Friedman. 1990. Production of tumor necrosis factor (TNF- $\alpha$ ) and lymphotoxin (TNF- $\beta$ ) by murine pre-B and B cell lymphomas. *J. Immunol.* 144:3424.
- Weil, D., and F. Dautry. 1988. Induction of tumor necrosis factor-alpha and -beta and interferon-gamma mRNA by interleukin 2 in murine lymphocytic cell lines. *Oncogene Res.* 3:409.
- Matsuhashi, M., Y.-X. Fu, H. Molina, and D. D. Chaplin. 1997. Lymphotoxin- $\alpha$ -deficient mice and TNF receptor-1-deficient mice define developmental and functional characteristics of germinal centers. *Immunol. Rev.* 156:137.
- Koni, P. A., R. Saccà, P. Lawton, J. L. Browning, N. H. Ruddle, and R. A. Flavell. 1997. Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin-beta deficient mice. *Immunity* 6:491.
- Hochman, P. S., G. R. Majeau, F. Mackay, and J. L. Browning. 1996. Proinflammatory responses are efficiently induced by homotrimeric but not heterotrimeric lymphotoxin ligands. *J. Inflamm.* 46:220.
- Browning, J. L., K. Miatkowski, I. Sizing, D. A. Griffiths, M. Zafari, C. D. Benjamin, W. Meier, and F. Mackay. 1996. Signaling through the lymphotoxin-beta receptor induces the death of some adenocarcinoma tumor lines. *J. Exp. Med.* 183:867.
- Browning, J. L., I. Dougas, A. Ngam-ek, P. R. Bourdon, B. N. Ehrenfels, K. Miatkowski, M. Zafari, A. M. Yampaglia, P. Lawton, W. Meier, C. P. Benjamin, and C. Hession. 1995. Characterization of surface lymphotoxin forms: use of specific monoclonal antibodies and soluble receptors. *J. Immunol.* 154:33.
- Browning, J. L., I. Dougas-Sizing, P. Lawton, P. Bourdon, P. D. Rennert, G. R. Majeau, C. M. Ambrose, C. Hession, K. Miatkowski, D. A. Griffiths, A. Ngam-ek, W. Meier, C. D. Benjamin, and P. S. Houchman. 1997. Characterization of lymphotoxin- $\alpha\beta$  complexes on the surface of mouse lymphocytes. *J. Immunol. In press this issue.*
- Browning, J., and A. Ribolini. 1989. Studies on the differing effects of tumor necrosis factor and lymphotoxin on the growth of several human tumor lines. *J. Immunol.* 143:1859.
- Chai, H., S. G. Vasudevan, A. G. Porter, K. L. Chua, S. Oh, and M. Yap. 1993. Glycosylation and high-level secretion of human tumour necrosis factor-beta in recombinant baculovirus-infected insect cells. *Biotechnol. Appl. Biochem.* 18:259.
- Crowe, P. D., T. L. VanArsdale, B. N. Walter, K. M. Dahms, and C. F. Ware. 1994. Production of lymphotoxin (LT alpha) and a soluble dimeric form of its receptor using the baculovirus expression system. *J. Immunol. Methods* 168:79.
- Sheehan, K. C. F., N. H. Ruddle, and R. D. Schreiber. 1989. Generation and characterization of hamster monoclonal antibodies that neutralize tumor necrosis factors. *J. Immunol.* 142:3884.
- Aggarwal, B. B., W. J. Henzel, B. Moffat, W. J. Kohn, and R. N. Harkins. 1985. Primary structure of human lymphotoxin derived from 1788 lymphoblastoid cell line. *J. Biol. Chem.* 260:2334.
- Schoenfeld, H. J., B. Poeschl, J. R. Frey, H. Loetscher, W. Hunziker, A. Lustig, and M. Zulauf. 1991. Efficient purification of recombinant human tumor necrosis factor beta from *Escherichia coli* yields biologically active protein with a trimeric structure that binds to both tumor necrosis factor receptors. *J. Biol. Chem.* 266:3863.
- Hains, J. M., and B. B. Aggarwal. 1989. Characterization of recombinant human lymphotoxin (tumor necrosis factor-beta) produced by a mammalian cell line. *Arch. Biochem. Biophys.* 274:417.
- Medvedev, A. E., T. Espvik, G. Ranges, and A. Sundan. 1996. Distinct roles of the two tumor necrosis factor (TNF) receptors in modulating TNF and lymphotoxin alpha effects. *J. Biol. Chem.* 271:9778.
- Medvedev, A. E., A. Laegreid, A. Sundan, and T. Espvik. 1996. A non-competitive P55 TNF receptor antibody enhances the specific activity of lymphotoxin-alpha. *Scand. J. Immunol.* 43:439.
- Laster, S. M., J. G. Wood, and L. R. Gooding. 1988. Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. *J. Immunol.* 141:2629.
- Nedwin, G. E., L. P. Svedersky, T. S. Bringman, M. A. Palladino, and D. V. Goeddel. 1985. Effect of interleukin-2, interferon- $\gamma$ , and mitogens on the production of tumor necrosis factors  $\alpha$  and  $\beta$ . *J. Immunol.* 135:2492.
- Hackett, S. P., and D. L. Stevens. 1993. Superantigens associated with staphylococcal and streptococcal toxic shock syndrome are potent inducers of tumor necrosis factor-beta synthesis. *J. Infect. Dis.* 168:232.
- Steffen, M., O. G. Ottmann, and M. A. Moore. 1988. Simultaneous production of tumor necrosis factor- $\alpha$  and lymphotoxin by normal T cells after induction with IL-2 and anti-T3. *J. Immunol.* 140:2621.
- Ruddle, N. H., C. M. Bergman, K. M. McGrath, E. G. Lingenheld, M. L. Grunnel, S. J. Padula, and R. B. Clark. 1990. An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. *J. Exp. Med.* 172:1193.
- Yang, X. D., R. Tisch, S. M. Singer, Z. A. Cao, R. S. Liblau, R. D. Schreiber, and H. O. McDevitt. 1994. Effect of tumor necrosis factor alpha on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. *J. Exp. Med.* 180:995.
- Smith, C. A., F.-Q. Hu, T. D. Smith, C. L. Richards, P. Smolak, R. G. Goodwin, and D. J. Pickup. 1996. Cowpox virus genome encodes a second soluble homologue of cellular TNF receptors distinct from CrmB, that binds TNF and not  $LT\alpha$ . *Virology* 223:132.

34. Banner, D. W., A. D'Arcy, W. Janes, R. Gentz, H. J. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer. 1993. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* 73:431.

35. Mackay, F., G. R. Majeau, P. S. Hochman, and J. L. Browning. 1996. Lymphotoxin- $\beta$  receptor triggering induces the activation of NF- $\kappa$ B transcription factor in some cell types. *J. Biol. Chem.* 271:24934.

36. Schuchmann, M., S. Hess, P. Butler, C. Brakebusch, D. Wallach, A. Porter, G. Riethmuller, and H. Engelmann. 1995. Functional discrepancies between tumor necrosis factor and lymphotoxin alpha explained by trimer stability and distinct receptor interactions. *Eur. J. Immunol.* 25:2183.

37. Estrov, Z., R. Kurzrock, E. Pocsik, S. Pathak, H. M. Kantarjian, T. F. Zif, D. Harris, M. Talpaz, and B. B. Aggarwal. 1993. Lymphotoxin is an autocrine growth factor for Epstein-Barr virus-infected B cell lines. *J. Exp. Med.* 177:763.

38. De Togni, P., J. Goellner, N. H. Ruddle, P. R. Streeter, A. Fick, S. Mariathasan, S. C. Smith, R. Carlson, L. P. Shornick, J. Strauss-Schoenberger, J. H. Russell, R. Karr, and D. D. Chaplin. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 264:703.

39. Banks, T. A., B. T. Rouse, M. K. Kerley, P. J. Blair, V. L. Godfrey, N. A. Kuklin, D. M. Bouley, J. Thomas, S. Kanangat, and M. L. Mucenski. 1995. Lymphotoxin- $\alpha$ -deficient mice: effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155:1685.

40. Eugster, H.-P., M. Mueller, U. Karrer, B. D. Car, B. Schnyder, V. M. Eng, G. Woerly, M. Le Hir, F. di Padova, M. Aguet, R. Zinkernagel, H. Bluthmann, and B. Ryffel. 1996. Multiple abnormalities in tumor necrosis factor and lymphotoxin- $\alpha$ -double deficient mice. *Int. Immunol.* 8:23.

41. Neumann, B., A. Luz, K. Pfeffer, and B. Holzmann. 1996. Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. *J. Exp. Med.* 184:259.

42. Pasparakis, M., L. Alexopoulou, V. Episkopou, and G. Kollias. 1996. Immune and inflammatory responses in TNF- $\alpha$  deficient mice: a critical requirement for TNF $\alpha$  in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers and in the maturation of the humoral immune response. *J. Exp. Med.* 184:1397.

43. Rennert, P. D., J. L. Browning, R. Mebius, F. Mackay, and P. S. Hochman. 1996. Surface lymphotoxin alpha/beta complex is required for the development for peripheral lymphoid organs. *J. Exp. Med.* 184:1999.

44. Rennert, P. D., J. L. Browning, and P. S. Hochman. 1997. Lymphotoxin ligands selectively function to generate and organize secondary lymphoid tissue. *Int. Immunol.* In press.

# Signaling through the Lymphotoxin $\beta$ Receptor Induces the Death of Some Adenocarcinoma Tumor Lines

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## Summary

Surface lymphotoxin (LT) is a heteromeric complex of LT- $\alpha$  and LT- $\beta$  chains that binds to the LT- $\beta$  receptor (LT- $\beta$ -R), a member of the tumor necrosis factor (TNF) family of receptors. The biological function of this receptor-ligand system is poorly characterized. Since signaling through other members of this receptor family can induce cell death, e.g., the TNF and Fas receptors, it is important to determine if similar signaling events can be communicated via the LT- $\beta$ -R. A soluble form of the surface complex was produced by coexpression of LT- $\alpha$  and a converted form of LT- $\beta$  wherein the normally type II LT- $\beta$  membrane protein was changed to a type I secreted form. Recombinant LT- $\alpha_1/\beta_2$  was cytotoxic to the human adenocarcinoma cell lines HT-29, WiDr, MDA-MB-468, and HT-3 when added with the synergizing agent interferon (IFN)  $\gamma$ . When immobilized on a plastic surface, anti-LT- $\beta$ -R monoclonal antibodies (mAbs) induced the death of these cells, demonstrating direct signaling via the LT- $\beta$ -R. Anti-LT- $\beta$ -R mAbs were also identified that inhibited ligand-induced cell death, whereas others were found to potentiate the activity of the ligand when added in solution. The human WiDr adenocarcinoma line forms solid tumors in immunocompromised mice, and treatment with an anti-LT- $\beta$ -R antibody combined with human IFN- $\gamma$  arrested tumor growth. The delineation of a biological signaling event mediated by the LT- $\beta$ -R opens a window for further studies on its immunological role, and furthermore, activation of the LT- $\beta$ -R may have an application in tumor therapy.

The TNF family of ligands and receptors is a set of regulatory elements in the immune system (1). TNF was discovered as a cytolytic agent circulating in the blood of endotoxin-stimulated animals (2-4). Originally cloned in the expectation that TNF would be a novel antitumor agent, it was later shown that its primary physiologic function lies in initiating the inflammatory cascade underlying the host's immediate defensive response to infection or stress. More complex immunological functions have been described (5, 6). Lymphotoxin (LT)<sup>1</sup>  $\alpha$  (also called TNF- $\beta$ ) is a similar cytokine secreted by activated lymphocytes (7) and was originally characterized as having the same functions as TNF. Later, activated T and B cells were found to display LT- $\alpha$  on their surfaces in an unusual form complexed with another member of the TNF family called LT- $\beta$  in an LT- $\alpha_1/\beta_2$  stoichiometry (8-13). A complex with an apparent

LT- $\alpha_2/\beta_1$  stoichiometry is also present, but only in minor amounts on human lymphocytes. The major LT- $\alpha_1/\beta_2$  form does not bind to the known TNF receptors, referred to here as TNF-R55 and TNF-R75, but rather interacts with another receptor in the TNF family called the LT- $\beta$  receptor (LT- $\beta$ -R) (9, 14).

Currently, the function of the LT system is poorly characterized, however, there are suggestions that LT signaling is involved in the development of the peripheral lymphoid organs. Genetic disruption of the LT- $\alpha$  gene in mice led to an unusual phenotype. The mice lacked lymph nodes and lost the organization of T and B cells in the follicles in the spleen (15, 16). A similar loss of lymph nodes occurs in *aly* mice although this mouse, unlike the LT- $\alpha$  knockout mouse, is severely immunocompromised (17). Signaling through the two known TNF receptors has not been shown to mediate the development of the lymph nodes since knockout of either receptor does not lead to the loss of lymph nodes (18, 19). Thus, it has been postulated that signaling through the LT- $\beta$ -R constitutes a regulatory pathway that is distinct from TNF-related events and may

<sup>1</sup> Abbreviations used in this paper: LT, lymphotoxin; LT- $\beta$ -R, LT- $\beta$  receptor; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase UTP nick-end labeling; VCAM, vascular cell adhesion molecule.

account for the unique phenotype of the LT- $\alpha$  knockout mouse (12, 15, 16).

Activation of several members of the TNF family of receptors can have cytotoxic or growth-inhibitory consequences (1). For example Fas receptor activation results in apoptosis of many cell types, including both transformed and non-transformed cells (20, 21), and this process is likely to play a role in the deletion of autoreactive lymphocytes in the periphery (22). TNF and LT- $\alpha$  also can kill some transformed cells, and it is likely that tumor cells respond abnormally by either necrosis or apoptosis to what is normally a differentiation-like signal. More recently, TNF signaling has been proposed to induce the death of nontransformed lymphoblasts in a slow fashion (23, 24), and this process appears to require TNF-R75. The physiological significance of this event remains to be explored. The Fas receptor and the TNF-R55 both possess a unique cytoplasmic domain, called the death domain, that is required to initiate cell death (25). CD30 and CD40 signaling can inhibit growth and may also induce apoptosis, yet these receptors as well as the TNF-R75 and the LT- $\beta$ -R lack obvious death domains (12, 26, 27). We have investigated whether LT- $\beta$ -R signaling could induce cell death both because of its possible immunological relevance and to provide a practical starting point for studying the role of the LT system. Using either recombinant ligands or antireceptor mAbs with agonist activity, the ability of LT- $\beta$ -R activation to induce cell death in various transformed lines was examined. In this report, we show that LT- $\beta$ -R signaling can induce cell death in a limited group of adenocarcinoma tumor lines.

## Materials and Methods

**Cells.** All cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD) except for WEHI 164 clone 13, which was obtained from Dr. Eric Kawashima (Glaxo Institute for Molecular Biology, Geneva, Switzerland). WEHI 164 cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS). HT-29 and WiDr cells were maintained in MEM with Earle's salts, 10% FCS with glutamine, penicillin/streptomycin, non-essential amino acids, and sodium pyruvate. These two cell lines are thought to be derived from the same patient (28). In our assays, the original ATCC HT-29 line was heterogeneous in its response to LT- $\alpha$ / $\beta$ , and not all of the cells died in a parallel manner. Subclones from the line were isolated by limiting dilution, and the HT-29-14 line was one subclone that behaved homogeneously in these assays. All of the results can be reproduced qualitatively with the parental line.

**Materials.** The anti-Fas mAb CH11 was obtained from Kamiya Biomedical Co. (Thousand Oaks, CA), the control IgG1 mouse mAb MOPC 21 from Organon Technica (Durham, NC) and the anti-CD40 mAb BB20 from R&D Systems (Minneapolis, MN). The anti-human LFA-3 mAb 1E6 has been described (29) and the anti-LFA-3 mAb TS2/9 was provided by Barbara Wallner. The anti-TNF mAb 104c has been described (30). The HT-29/26 hybridoma that produces a mAb that recognizes an abundant antigen on the HT-29 surface was obtained from ATCC, cells were grown, and the mAb purified by protein A-Sepharose chromatography. The LT- $\beta$ -R-hIgG1 and TNF-R55-hIgG1 Fc fusion proteins have been described (9).

**Recombinant Cytokines.** Human TNF and IFN- $\gamma$  were produced at Biogen (30). Recombinant human LT- $\alpha$  was prepared by expression in insect cells as described (31) and was similar to material expressed in CHO cells (30). The recombinant LT- $\alpha$ / $\beta$  heteromeric forms were prepared by coinfection of insect cells with two baculoviruses encoding the human LT- $\alpha$  and human LT- $\beta$  proteins (Browning, J.L., K. Miatkowski, D.A. Griffiths, P.R. Bourdon, C. Hession, C.M. Ambrose, and W. Meier, manuscript in preparation). The transmembrane region of the LT- $\beta$  gene was replaced with a vascular cell adhesion molecule (VCAM) leader sequence to enable secretion of mixed LT- $\alpha$ / $\beta$  forms. The trimers LT- $\alpha_1$ / $\beta_2$ , LT- $\alpha_2$ / $\beta_1$ , and LT- $\alpha_3$  were purified using combinations of p55 TNF-R and LT- $\beta$ -R affinity columns. The resultant preparations have been well characterized, contain only LT forms, are trimeric, and are >95% pure with respect to LT forms based on ion exchange chromatographic resolution of the three stoichiometrically different trimers. The LT- $\alpha$ / $\beta$  preparation contained <1 part in 5,000 of LT- $\alpha_3$ -like activity as assessed using the WEHI 164 indicator line and by comparison of various LT trimers prepared with a LT- $\alpha$  D50N mutation that eliminates TNF-R binding (Browning J.L., K. Miatkowski, D.A. Griffiths, P.R. Bourdon, C. Hession, C.M. Ambrose, and W. Meier, manuscript in preparation).

**Cytotoxicity Assays.** In the cytotoxicity assays, serial dilutions of the cytokines or antibodies were prepared in 50  $\mu$ l in 96-well plates, and 5,000 HT-29-14 cells were added in 50  $\mu$ l of media with or without IFN- $\gamma$ . After 3–4 d, 10  $\mu$ l of 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide] was added, and after 3 h the formazan was dissolved by adding 100  $\mu$ l of 10% SDS in 10 mM HCl. After a further 24-h incubation at 37°C, the OD was quantitated at 550 nm. In some experiments, soluble receptor forms or pure human IgG were added in 10  $\mu$ l before the addition of the cells. To immobilize mAbs on the plastic surface, 96-well tissue culture plates were first coated with 50  $\mu$ l of 10  $\mu$ g/ml affinity-purified goat anti-mouse Fc polyclonal antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), washed with 5% FBS in PBS, and then coated with varying amounts of the various mAbs diluted into tissue culture media with FBS. The plates were washed with media before use. Cells were added and growth assessed as indicated above. To survey the panel of cells shown in Table 4, cells were plated in the presence of 50 U/ml IFN- $\gamma$  with various dilutions of TNF, anti-Fas, LT- $\alpha$ / $\beta$ , or into wells coated with various anti-LT- $\beta$ -R mAbs as described above. Growth was assessed with the MTT readout, and wells were also visually inspected for morphology changes. Dramatic growth inhibition was scored as two pluses (++)+, partial growth inhibition at reasonable concentrations was noted as one plus (+), and partial effects requiring high concentrations of ligand was marked as plus/minus (+/-).

**Mouse Anti-LT- $\beta$ -R mAbs.** Mouse hybridomas producing mAbs to the human LT- $\beta$ -R were prepared by immunization of RBF mice with the LT- $\beta$ -R-Fc chimera essentially as described previously (9). All mAbs were IgG1 isotypes.

**FACS® Binding Assays.** To monitor receptor binding to surface ligand, 200 ng/ml soluble LT- $\beta$ -R-Fc were added to PMA-activated II-23 T cell hybridoma cells, and binding was detected using a PE-labeled donkey anti-human Ig essentially as described (9). To assess the blocking ability of the mAbs, mAbs were preincubated with the soluble receptor for 20 min before addition of the receptor-mAb mixture to the II-23 cells. In other experiments, receptor expression on adherent tumor lines was determined by FACS® analysis of cells removed with PBS with 5 mM EDTA and stained using anti-LT- $\beta$ -R mAbs and a PE-labeled

donkey anti-mouse IgG reagent (Jackson ImmunoResearch Laboratories).

**Epitope Mapping by BIAcore™ Analysis.** Affinity-purified goat anti-human Fc (Jackson ImmunoResearch Laboratories) was immobilized onto an *N*-hydroxysuccinimide-activated sensor chip, and LT- $\beta$ -R-Fc was captured onto the anti-human-Fc-coated chip. The various pairs of anti-LT- $\beta$ -R mAbs were then bound sequentially, and the ability of the second mAb to bind in the presence of the first mAb was measured using a BIAcore™ 2000 (Pharmacia Biosensor, Uppsala, Sweden). The entire array of 49 mAb combinations were assessed for cross-blocking in this manner and analyzed essentially as described (32).

**Analysis of Apoptosis.** Terminal deoxynucleotidyl transferase UTP nick-end labeling (TUNEL) of free DNA ends, i.e., TUNEL staining, was carried out using the ApopTag™ kit (Oncor Inc., Gaithersburg, MD) according to the manufacturer's specifications.

**Tumor Growth in SCID Mice.** BALB/c SCID female mice at 6–8 wk old (The Jackson Laboratory, Bar Harbor, ME) were injected with  $10^6$  trypsinized and washed WiDr cells in a volume of 0.2 ml of PBS subcutaneously onto the back of the animal. Mice were treated with or without antibody either with or without  $10^6$  antiviral U/mouse of human IFN- $\gamma$  by intraperitoneal injection in 0.2 ml on days 0 and 1 or as indicated. The amounts of IFN- $\gamma$  and antibody have not been optimized. Tumor volume was calculated from the radius as determined by caliper measurements in two dimensions. The results shown in Fig. 6 B were determined in a blinded format.

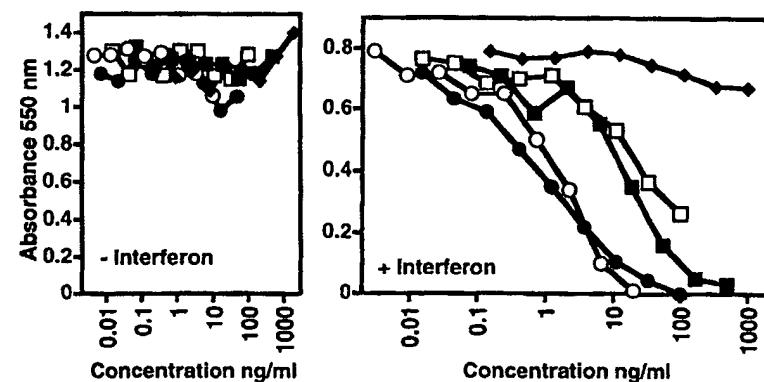
## Results

**LT- $\alpha$ / $\beta$ <sub>2</sub> Is Cytotoxic to HT-29 Cells.** Recombinant LT- $\alpha$ / $\beta$ <sub>2</sub> and LT- $\alpha$ <sub>2</sub>/ $\beta$ <sub>1</sub> trimers were tested for their ability to inhibit the growth of a number of tumor lines. IFN- $\gamma$  was included in the screening as it had been shown to enhance the cytolytic properties of TNF (33). Recombinant soluble LT- $\alpha$ / $\beta$ <sub>2</sub> inhibited the growth of human HT-29 cells only in the presence of IFN- $\gamma$  (Fig. 1) and, as shown previously, this cell line was also sensitive to the anti-Fas receptor mAb CH11, TNF, and LT- $\alpha$  (30, 34, 35). IFN- $\alpha$  and - $\beta$  were 100-fold less effective when compared on the basis of anti-viral units (data not shown). Soluble LT- $\alpha$ <sub>2</sub>/ $\beta$ <sub>1</sub> was much less active in this assay.

The specificity of the LT- $\alpha$ / $\beta$ <sub>2</sub> cytotoxicity was examined in several ways. Soluble TNF-R55 and LT- $\beta$ -R immunoglobulin chimeras (TNF-R55-Fc and LT- $\beta$ -R-Fc)

were tested for their ability to block the various activities (9). These receptors can bind to the appropriate cleft between two subunits in the trimeric ligand structures and interfere with the ability of receptor on the membrane to bind ligand. As expected and shown previously (36, 37), TNF-R55-Fc completely blocked TNF-induced growth inhibition by binding to TNF and preventing its interaction with surface receptor (Table 1). Soluble TNF-R had no effect on LT- $\alpha$ / $\beta$ <sub>2</sub>-mediated antiproliferative effects. In contrast, LT- $\beta$ -R-Fc blocked LT- $\alpha$ / $\beta$ <sub>2</sub> effects, but not those of TNF or LT- $\alpha$ . Moreover, anti-LT- $\alpha$ -neutralizing mAbs (9) did not affect the LT- $\alpha$ / $\beta$ <sub>2</sub> cytotoxicity (data not shown), confirming that soluble trace LT- $\alpha$  contaminants were not involved in the activity of LT- $\alpha$ / $\beta$ <sub>2</sub> on HT-29 cells. Additionally, a mutated form of LT- $\alpha$  (D50N) that lacks the ability to signal through the TNF-R was examined (38). LT- $\alpha$ / $\beta$ <sub>2</sub> prepared with the mutant LT- $\alpha$  retained essentially full activity on HT-29 and WiDr cells (see below), further eliminating TNF-R55 binding as a possible mechanism for the cytotoxic effects of LT- $\alpha$ / $\beta$ <sub>2</sub> (Ambrose, C., unpublished data). An anti-TNF neutralizing mAb 104c, also had no effect on LT- $\alpha$ / $\beta$ <sub>2</sub> activity, precluding the induction of TNF synthesis as a mechanism for the LT- $\alpha$ / $\beta$ <sub>2</sub> effects. These assays indicate that LT- $\alpha$ / $\beta$ <sub>2</sub> can trigger cytotoxic events via non-TNF-R-mediated mechanisms.

**Mechanism of Growth Inhibition.** The growth inhibition assay alone does not discriminate between death and stasis; however, direct observation of the treated HT-29 cells showed that Fas receptor activation led to rapid cell death, i.e., within 12–24 h, whereas TNF effects were slower and required  $\sim$ 24 h. LT- $\alpha$ / $\beta$ <sub>2</sub>-treated cells underwent a much slower death, with dead cells not being visible until 1.5–2 d. The morphology of the dying cells was identical in all three cases. Some cells have the appearance of apoptotic bodies with condensed nuclei, whereas in others the nucleus appears to condense and the cytoplasm balloons out and becomes clear (Fig. 2). Substantial cell lysis does not occur even after 3–4 d. The TNF-, anti-Fas receptor mAb- or LT- $\alpha$ / $\beta$ <sub>2</sub>-treated populations had more cells that stained brightly with HOECHST dye staining (data not shown), which can be indicative of chromatin condensation accompanying apoptotic events. Internucleosomal DNA fragmen-



**Figure 1.** The cytotoxic effects of LT- $\alpha$ / $\beta$ <sub>2</sub> on the human adenocarcinoma HT-29 cells. Comparison of the cytotoxic activity of anti-Fas receptor mAb CH-11 (●), TNF (○), LT- $\alpha$  (□), LT- $\alpha$ / $\beta$ <sub>2</sub> (■), and LT- $\alpha$ <sub>2</sub>/ $\beta$ <sub>1</sub> (◆) in the presence and absence of 80 U/ml IFN- $\gamma$ .

**Table 1. Ability of LT- $\beta$ -R and TNF-R55 Immunoglobulin Fusion Proteins to Block the Inhibitory Effects of Various TNF and LT Ligands on HT-29 Growth**

Cytotoxic agent	Concentration of cytotoxic agent resulting in 50% growth inhibition		
	Presence of*		
	hu-IgG control	TNF-R55-Fc	LT- $\beta$ -R-Fc
TNF	0.08	>10 <sup>‡</sup>	0.08
LT- $\alpha$	3.0	>1000	3.0
LT- $\alpha_1/\beta_2$	5.0	5.0	>200.0
Anti-Fas mAb	2.0	2.0	2.0

\*Each cytotoxic agent was premixed with the Ig fusion proteins for 10 min before addition to the cells. The final concentration of fusion protein was 5  $\mu$ g/ml.

<sup>‡</sup>Higher concentrations were not tested.

tation or laddering was not observed after LT- $\alpha_1/\beta_2$ , TNF, or anti-Fas treatment of HT-29 or WiDr cells, although a prominent large DNA band was visible (data not shown) after all three treatments. This large fragment is reminiscent of the large 50–200-kb cleavage products previously described in dying epithelial tumor cells (39, 40). Some DNA fragmentation was observed using TUNEL staining of 3' hydroxyl ends of DNA, which is more sensitive than DNA laddering (Fig. 3). Therefore, the death induced by TNF-R, Fas, or LT- $\beta$ -R signaling may be basically apoptotic even though all of the classic features of apoptosis have not been observed in epithelial tumors (39, 41).

**Properties of Anti-LT- $\beta$ -R mAbs.** Antibodies to receptors in the TNF family can have either antagonistic or agonistic effects, and as tools they have been very useful in delineating the consequences of receptor activation. To determine if LT- $\beta$ -R cross-linking could induce cell death, we prepared and characterized similar LT- $\beta$ -R-specific mAbs. Mice were immunized with the LT- $\beta$ -R-Fc fusion protein, and a panel of mouse anti-LT- $\beta$ -R mAbs were isolated. These mAbs were grouped into four subsets based on their performance in the following assays: (a) the ability to cross-block each other in a mAb/antigen-binding experiment using plasmon resonance detection (i.e., a BIAcore<sup>TM</sup> epitope mapping experiment); (b) the ability to block soluble LT- $\beta$ -R-Fc binding to surface ligand on PMA-activated II-23 cells; and (c) the ability to affect LT- $\alpha_1/\beta_2$ -induced cell death. The results of this analysis are summarized in Table 2.

Epitope mapping using the BIAcore<sup>TM</sup> instrument defined four separate epitopes recognized by this panel of mAbs and formed the basis of the grouping shown in Table 2. Within each group, the mAbs effectively blocked each other. The group I mAbs did not cross-block any other mAbs. The group II epitope partially overlapped the group III epitope. We identified only one mAb, CBE11, with group IV properties, and its epitope slightly overlapped the

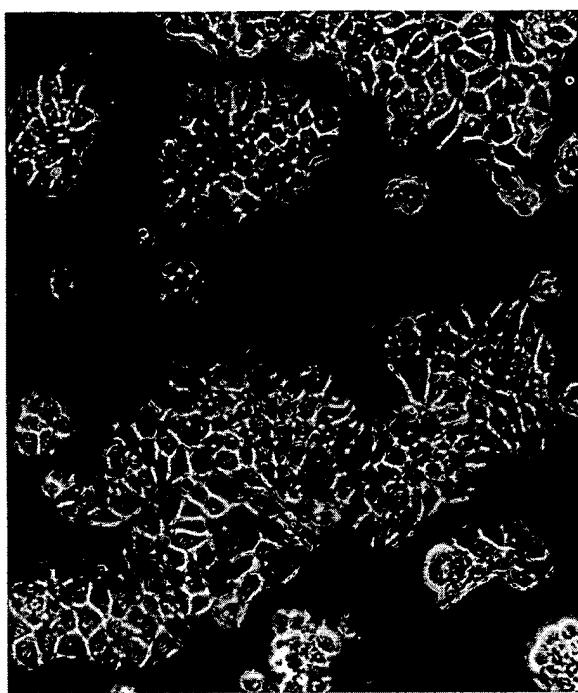
BCG6 and BKA11 sites. To directly assess the ability of the mAbs to inhibit receptor-ligand binding, a FACS<sup>®</sup> binding assay was used. Antibodies were premixed with soluble receptor, and the ability of the receptor to bind to ligand on the surface of II-23 cells was quantitated. This assay has the advantage that any agonistic activity of the antireceptor mAbs would not be apparent. mAbs from groups I, II, and IV were effective inhibitors, suggesting that they bind close to the ligand binding region of the receptor (Table 2).

In the HT-29 cytotoxicity assay, all mAbs in these groups either weakly inhibited growth or lacked activity when simply added to the culture in the absence of ligand (Fig. 4 A). There was considerable variability in this assay possibly reflecting differing receptor densities at the start of the experiment; e.g., compare Fig. 4 A and Table 3. In dramatic contrast, when the mAbs were immobilized on the plastic, all mAbs were found to have potent cytotoxic activity (Fig. 4 B), and this activity was again completely dependent on the presence of IFN- $\gamma$ . This result demonstrates that LT- $\beta$ -R cross-linking is sufficient to induce cell death. Moreover, we reasoned that mAbs to two different epitopes should be able to cross-link receptors more effectively, and indeed Table 3 shows that certain pairs of mAbs were cytotoxic in solution. In general, mAbs from two different groups had to be paired to get cytotoxic activity.

In addition to the direct agonistic effects of the mAbs described above, we investigated the effects of the mAbs on ligand induced cell death. Each of the four groups of mAbs had differing effects reinforcing the grouping based on BIAcore<sup>TM</sup> epitope mapping. Group I mAbs primarily blocked LT- $\alpha_1/\beta_2$  activity with a small amount of direct growth inhibition occurring in the absence of ligand (Fig. 4 C). In some experiments (data not shown), a Fab fragment of BDA8 was used, and the small growth inhibitory activity disappeared and only direct inhibition was observed. Such complex mixed agonist/antagonist effects have been observed with anti-CD40 mAbs (42). The group II mAbs had complex effects that suggested mixed agonistic and antagonistic behavior (data not shown). Group III mAbs potentiated LT- $\alpha_1/\beta_2$  activity presumably by creating local regions of high receptor density that would enhance ligand mediated cross-linking (Fig. 4 D), and there was no evidence of antagonistic effects. The cytotoxic effects of TNF were also slightly potentiated by these mAbs, suggesting that certain signal transduction elements were facilitating or priming the cells for TNF signaling. The group IV mAb, CBE11, did not affect ligand-mediated cytotoxicity. Because the LT- $\beta$ -R-specific mAbs can directly effect death, one can conclude that the LT- $\beta$ -R is able to signal cell death, and this event accounts for the cytotoxic activity of LT- $\alpha_1/\beta_2$ .

**Survey of Sensitive Cell Lines.** A series of human tumor lines have been screened for sensitivity to cytotoxic or growth-inhibitory effects of either the LT- $\alpha_1/\beta_2$  ligand or plastic immobilized anti-LT- $\beta$ -R mAbs in the presence of IFN- $\gamma$  (summarized in Table 4). The WiDr line (43) was found to be sensitive to LT- $\alpha_1/\beta_2$  in a very similar manner as the HT-29 line, and it is likely that this line is actually a

## INTERFERON- $\gamma$



## INTERFERON- $\gamma$ + LT $\alpha_1/\beta_2$

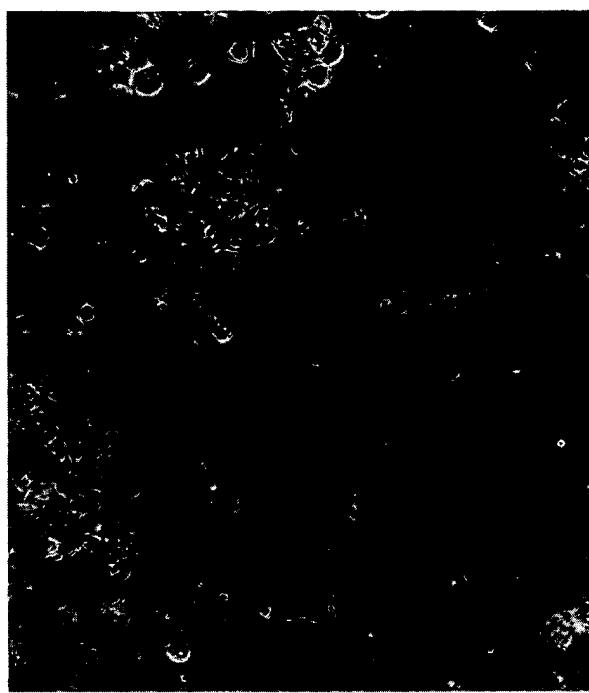


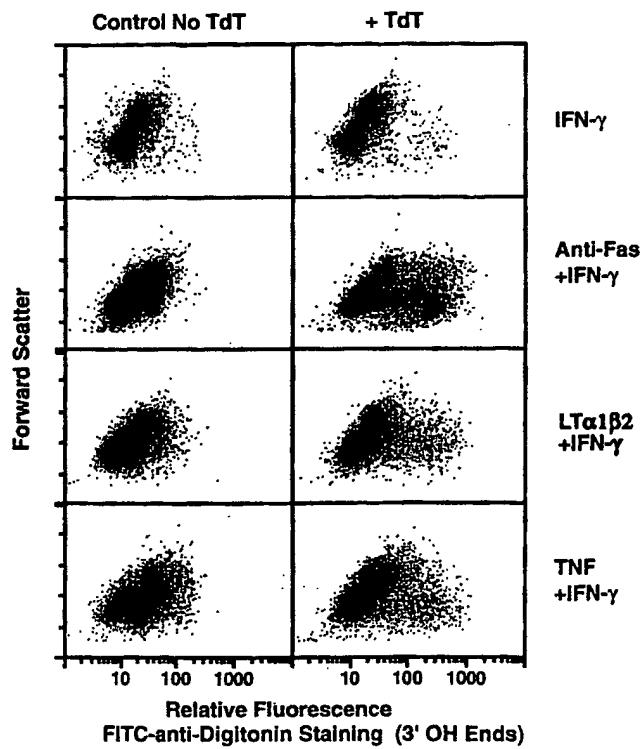
Figure 2. Photograph of HT-29 cells treated with IFN- $\gamma$  (80 U/ml) alone or IFN- $\gamma$  plus 50 ng/ml LT- $\alpha_1/\beta_2$  for 3 d  $\times 200$ .

derivative of HT-29 (28). The breast adenocarcinoma MDA-MB-468 and cervical carcinoma HT-3 lines were growth inhibited, and whether cell death occurred was not rigorously determined. In general, however, the majority of tumor lines are not sensitive under these relatively fixed conditions. This survey was complicated by the fact that the sensitivity of the cells to IFN- $\gamma$  varied dramatically, and the lines are often heterogeneous, with mixtures of responsive and nonresponsive cells obfuscating a simple MTT-based test.

Cell surface levels of LT- $\beta$ -R were examined to determine whether LT- $\beta$ -R presence is the determining factor for sensitivity to the LT ligand. Fig. 5 shows a comparison of LT- $\beta$ -R and CD40 receptor levels on four different tumor lines. The CD40 staining is included since its surface levels are very abundant on some epithelial tumor lines, as is the LT- $\beta$ -R. All four cell lines are LT- $\beta$ -R positive, and these results are typical of most nonlymphoid cells (Mackay, F., and J.L. Browning, unpublished results). Despite the presence of LT- $\beta$ -R on all four cell types, only HT-29 cells are appreciably sensitive to LT- $\alpha_1/\beta_2$ , indicating that sensitivity does not correlate simply with receptor presence. In the TNF, Fas, and CD40 signaling systems, no correlation has ever been observed between the level of surface receptor and whether a cell type responds biologically. Relative to Fas, TNF-R55, and TNF-R75, LT- $\beta$ -R is very abundant on cells. Experiments on the HT-29 and WiDr lines indicated that IFN- $\gamma$  treatment did not upregulate the LT- $\beta$ -R, whereas in the same experiments the Fas receptor was dramatically upregulated as described previously (35).

There was no evidence that LT- $\beta$ -R signaling was cytostatic to normal human fibroblasts, endothelial cells, or primary lymphocytes, which was expected since lymphocytes do not express the receptor (Hochman, P., J.L. Browning, and C. Ware, unpublished observations). The growth of the human diploid fibroblastoid line WI-38 was stimulated by LT- $\beta$ -R signaling (data not shown). The adenocarcinoma lines SW620 and SW1417 both displayed altered morphology in response to LT- $\beta$ -R signaling without growth arrest.

*Anti-LT- $\beta$ -R mAb Inhibits the Growth of WiDr in SCID Mice.* We have explored the ability of an anti-LT- $\beta$ -R mAb, CBE11, to block the growth of the WiDr line in immunodeficient mice. When the mice were treated intraperitoneally with the CBE11 mAb at the same time as the WiDr cells were inoculated subcutaneously, tumor outgrowth was dramatically blocked (Fig. 6 A). The antitumor action was enhanced by IFN- $\gamma$ ; however, the mAb was effective even without exogenous IFN- $\gamma$ . In the CBE11 + IFN- $\gamma$  group, 7 of 16 animals completely lacked tumors, whereas the remaining animals had small nodules that had not progressed at 2 mo. The CBE11 animals treated without IFN- $\gamma$  were similar to the CBE11 + IFN- $\gamma$  group at 30 d; however, these mice eventually developed slowly growing tumors. There were statistically significant differences between the control or IFN- $\gamma$ -treated groups and the CBE11-treated groups, whereas no significant differences were observed between the saline, IFN- $\gamma$ , or the control anti-human LFA3 mAb (1E6) + IFN- $\gamma$  groups. The 1E6 and CBE11 mAbs are both IgG1s, and since the 1E6 mAb effectively coats the tumor line yet did not block tumor



**Figure 3.** FACS<sup>®</sup> analysis of TUNEL staining of DNA fragmentation occurring in response to Fas, TNF, or LT- $\alpha_1/\beta_2$  signaling. HT-29 cells were exposed to 80 U/ml of IFN- $\gamma$  for 3 d, 50 ng/ml anti-Fas mAb and IFN- $\gamma$  for 2 d, 50 ng/ml LT- $\alpha_1/\beta_2$  and IFN- $\gamma$  for 3 d, or 10 ng/ml TNF with IFN- $\gamma$  for 2 d. All cells in the culture were stained using the Apo-Tag<sup>TM</sup> kit (Oncor, Inc.) without or with terminal deoxynucleotidyl transferase addition.

**Table 2. Summary of Mouse Anti-Human LT- $\beta$ -R mAbs**

mAb group	mAb name	Cell staining*	Blocking receptor binding†	HT-29 cytotoxicity		
				mAb immobilized on Plastic§	Soluble mAb alone	Soluble mAb with LT- $\alpha_1/\beta_1$
I	BDA8	+++	+++	+	+/-	Inhibits
I	AGH1	+++	+++	+	+/-	Inhibits
II	BCG6	+++	++	+	+/-	Mixed
II	BHA10	+++	+++	+	+/-	Mixed
III	BKA11	+++	+/-	+	-	Potentiates
III	CDH10	+++	+/-	+	+/-	Potentiates
IV	CBE11	+++	+++	+	+/-	No effect
Controls	MOPC21	-	-	-	-	No effect
	HT29/26	-	ND	-	-	No effect
	TS 2/9†	ND	ND	-	-	No effect

\*FACS<sup>®</sup> staining of CHO cells transfected with LT- $\beta$ -R.

†Assay assessed whether antibody blocks binding of soluble receptor to the activated T cell hybridoma II-23.

§HT-29 cells were grown with IFN- $\gamma$  on anti-LT- $\beta$ -R-coated plates as described in Materials and Methods.

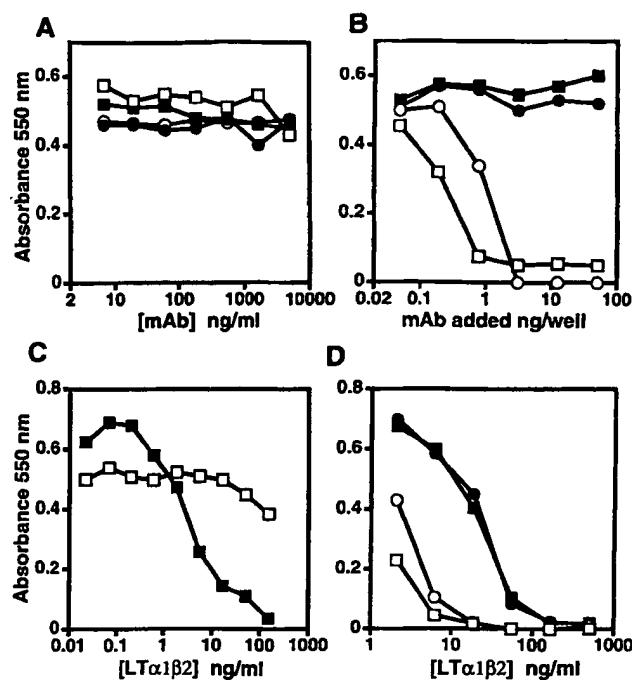
||Variable, partial inhibition in some assays, no effects in others.

†Anti-human LFA-3, a mouse IgG1.

growth, we can exclude complement- or NK cell-mediated events as the basis for the tumor inhibition. The efficacy of CBE11 in the absence of IFN- $\gamma$  was unexpected since there was an absolute dependence on IFN- $\gamma$  for any LT- $\beta$ -R based in vitro cytotoxic effect. Either there is some crossover of mouse IFN- $\gamma$  onto human IFN- $\gamma$  receptors, or other mechanisms are involved in vivo. The mechanism(s) by which LT- $\beta$ -R signaling prevents tumor growth in vivo effects are being investigated. The ability of combined IFN- $\gamma$ /CBE11 treatment to inhibit the growth of an established tumor was demonstrated (Fig. 6 B). Mice were inoculated with 10<sup>6</sup> WiDr cells, and after 15 d, treatment was initiated. At this point the average tumor volume was 0.076 cc or an average diameter of 0.532 cm. Treated tumors stopped growing, and after three injections of antibody over 3 wk, growth was arrested out to 7 wk after inoculation, at which point the experiment was terminated.

## Discussion

Activation of the receptors of the TNF family can direct cells into a proliferative or differentiation type response, or it can induce cell death sometimes even in the same cell type depending on the conditions (4, 22, 44). In this work, we have shown that signaling through the LT- $\beta$ -R leads to the death of the HT-29 and WiDr human adenocarcinoma cell lines and is at least growth inhibitory to two other lines. This activity represents the first observed effect of LT- $\beta$ -R signaling and is important not only because of the current interest in cytotoxic events, but because it provides a biological assay for characterizing various reagents. There was no evidence that LT- $\beta$ -R signaling was cytostatic to



**Figure 4.** Effects of various anti-LT- $\beta$ -R mAbs on HT-29 growth. *A* shows the effects of soluble anti-LT- $\beta$ -R mAbs alone on the growth of HT-29 cells; control IgG1 (●), HT-29/26, a mAb to an irrelevant abundant surface Ag on HT-29 cells (■), BDA8 (○), and CDH10 (□). *B* illustrates the direct cytotoxic effects of these two anti-LT- $\beta$ -R mAbs on HT-29 cells when immobilized on the plastic surface. Plates were coated with IgG1 (●), HT-29/26 (■), BDA8 (○), and CDH10 (□). *C* shows the LT- $\alpha_1/\beta_2$  antiproliferative effects on HT-29 cells in the presence of 2  $\mu$ g/ml control IgG1 (■) or anti-LT- $\beta$ -R mAb BDA8 (□). BDA8 exhibits some agonist activity even at low concentrations. *D* shows the effects of CDH10 as an example of a group III anti-LT- $\beta$ -R mAb that potentiates the effects of LT- $\alpha_1/\beta_2$ . LT- $\alpha_1/\beta_2$  effects were measured in the presence of no mAb (●), 0.5  $\mu$ g/ml control IgG1 (■), 0.05  $\mu$ g/ml CDH10 (○), and 0.5  $\mu$ g/ml (□) CDH10.

nontransformed cells, and signaling could actually drive growth in some situations. This pattern of cell sensitivity to LT- $\alpha_1/\beta_2$  basically resembles the effects of TNF. The data we have obtained suggest that the cytotoxicity mediated by the LT- $\beta$ -R will be limited to transformed cells. Recently, signaling through TNF-R75 has been reported to mediate an unusually slow death of nontransformed activated lymphocytes (23, 24), indicating that TNF-induced death may not be limited to transformed cells. In contrast, nontransformed lymphocytes can be induced to undergo rapid apoptosis after Fas signaling, and this event is most likely to be involved in the deletion of certain lymphocyte populations in the periphery (22, 45). Whether the slow events involving LT- $\beta$ -R or TNF-R75 are physiologically important will require further investigation. Surface LT is abundant on lymphokine activated T cells, i.e., LAK cells (12, 46). Currently, cell mediated cytotoxicity is thought to be mediated through the perforin and/or Fas pathways (47). A classic LT- $\beta$ -R-positive NK/LAK target, K562, was found to be completely resistant to the action of soluble LT- $\alpha_1/\beta_2$ , suggesting that neither surface LT is not involved in NK-like cytotoxicity. Genetic disruption of LT- $\alpha$  and the two

**Table 3.** Effect of Pairs of Anti-LT- $\beta$ -R mAbs on HT-29/WiDr Growth

mAb	Concentration (ng/ml)	mAb	Concentration (ng/ml)	Cell growth
IgG1	200	—	—	0.77 (HT-29)
BDA8	100	—	—	0.33*
—	—	BCG6	100	0.50
BDA8	100	BCG6	100	0.05
-----				
IGG1	50	—	—	0.85 (HT-29)
CDH10	33	—	—	0.525
—	—	AGH1	50	0.49
CDH10	33	AGH1	50	0.23
-----				
IgG1	100	—	—	0.80 (HT-29)
CDH10	10	—	—	0.86
IgG1	50	CDH10	10	0.81
—	—	CBE11	50	0.58
CDH10	10	CBE11	50	0.21
BHA10	10	—	—	0.85
BHA10	10	CBE11	50	0.09
-----				
IgG1	50	—	—	0.62 (WiDr)
CDH10	33	—	—	0.36
—	—	AGH1	50	0.36
CDH10	33	AGH1	50	0.16

Dotted lines indicate separate experiments.

\*Typically, further increases in the mAb concentration did not increase the amount of growth inhibition.

TNF-R does not impair CTL-mediated cell death supporting the hypothesis that TNF/LT signaling is not involved in cell-cell killing (13). Further experimental work will be required to determine whether LT signaling via direct cell-cell contact supports these conclusions.

The LT system with its heterotrimeric ligand is unusual. The cytotoxic activity resides primarily in the LT- $\alpha_1/\beta_2$  form, with the LT- $\alpha_2/\beta_1$  complex being much less active. The crystal structure of LT- $\alpha$  complexed with the TNF-R55 revealed that the receptor lies in the groove between two adjacent subunits (48). The higher potency of the LT- $\alpha_1/\beta_2$  form suggests that the  $\beta/\beta$  cleft, which is unique in the LT- $\alpha_1/\beta_2$  heterotrimer, must interact with the LT- $\beta$ -R. Biochemical analyses of this interaction have confirmed that there is a high affinity interaction of LT- $\beta$ -R with LT- $\alpha_1/\beta_2$  and a lower affinity interaction with LT- $\alpha_2/\beta_1$  (Browning, J.L., M. Zafari, C. Benjamin, W. Meier, D. Griffiths, and K. Miatkowski, unpublished observations). The exact nature of the signaling complex is currently unclear.

Antibodies to the TNF-R55 (49, 50), Fas receptor (21, 35, 51), CD27 (52), and CD40 (53) have been shown to have receptor-activating properties. Presumably antibodies

**Table 4. Summary of the Effects of TNF, Anti-Fas, LT- $\alpha_1/\beta_2$ , and Anti-LT- $\beta$ -R on the Growth of Various Cells in the Presence of IFN- $\gamma$** 

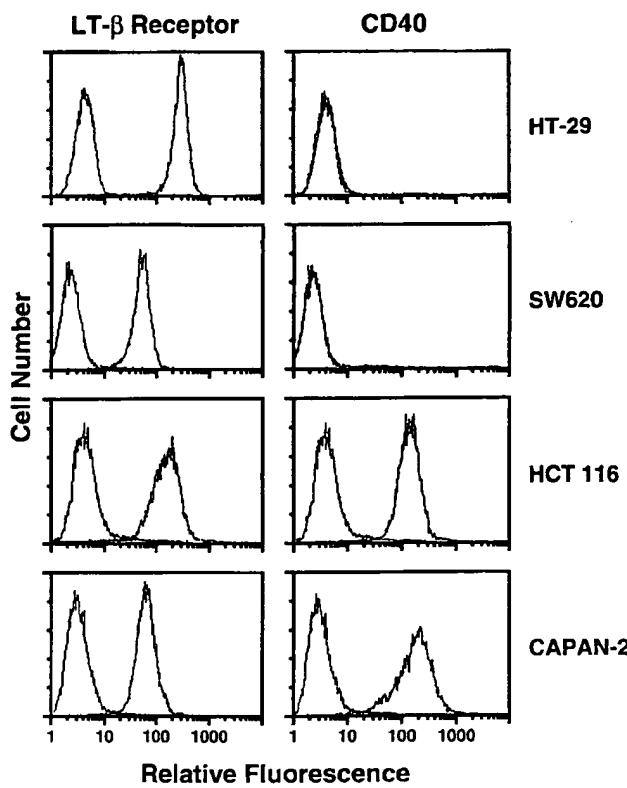
Name	Type	Growth inhibition by			
		TNF	Anti-Fas	LT- $\alpha_1/\beta_2$	Anti-LT- $\beta$ -R
<b>Brain</b>					
U118	Glioblastoma	+-	+-	-	-
SW1783	Astrocytoma	-	++	-	-
SW1088	Astrocytoma	+	+-	-	-
<b>Skin</b>					
A375	Melanoma	-	-	-	ND
SK-MEL-1	Melanoma	-	-	-	-
<b>Colorectal</b>					
HT29	Adenocarcinoma	++	++	++	++
WiDr	Adenocarcinoma	++	++	++	++
SK-Co-1	Adenocarcinoma	++	++	-	-
SW 403	Adenocarcinoma	-	++	-	-
SW 480	Adenocarcinoma	+	+	-	-
SW 620	Adenocarcinoma	-	-	*‡	-
SW 837	Adenocarcinoma	+-	+-	-	-
SW 1116	Adenocarcinoma	-	+	-	ND
SW 1417	Adenocarcinoma	-	-	‡	ND
Colo 320DM	Adenocarcinoma	-	ND	-	ND
LoVo	Adenocarcinoma	+	+-	-	-
DLD-1	Adenocarcinoma	+	+	-	+-
LS 174T	Adenocarcinoma	++	+-	-	-
LS 123	Adenocarcinoma	+	-	-	ND
T84	Carcinoma	+	++	-	-
HCT 116	Carcinoma	+-	-	-	ND
NCI H508	Adenocarcinoma	++	+	-	ND
CACO-2	Adenocarcinoma	-	-	-	-
<b>Breast</b>					
BT-20	Carcinoma	++	-	-	ND
SK-BR-3	Carcinoma	+-	-	+-‡\$	-
MCF-7	Adenocarcinoma	++	+	+-‡	ND
MDA-MB-468	Adenocarcinoma	++	ND	+-§	+
<b>Cervix</b>					
ME180	Carcinoma	+	+	+-	+
HT-3	Carcinoma	++	-	++‡	+-
MS751	Carcinoma	+-	+-	-	ND
<b>Ovary</b>					
SK-OV-3	Adenocarcinoma	-	-	-	-
<b>Pancreas</b>					
PANC-1	Epitheloid carcinoma	+	+	-	-
Capan-1	Adenocarcinoma	-	++	-	-
Capan-2	Adenocarcinoma	-	++	-	-
<b>Lung</b>					
A549	Carcinoma	+-	ND	-	ND
<b>Lymphoid</b>					
U937	Histiocytic	++	+-	+-	ND
K562	Promyelocytic	-	-	-	-

\*Some growth stimulation.

‡Altered morphology.

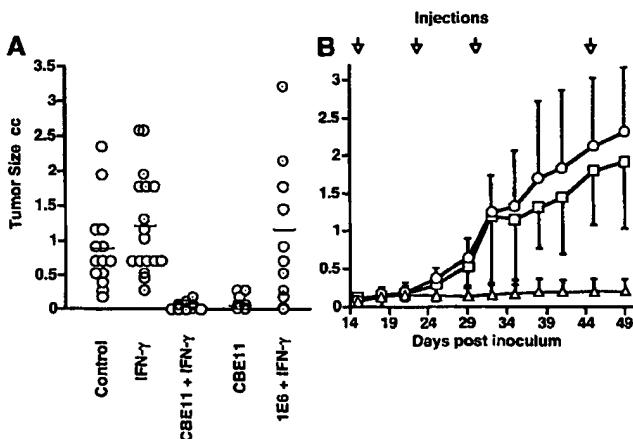
§Heterogenous cell population, responders and nonresponders present.

||Unconfirmed if LT- $\alpha$  component.



**Figure 5.** FACS<sup>®</sup> analysis comparing CD40 and LT- $\beta$ -R levels on three colorectal carcinoma lines, HT-29, SW620, HCT 116, and the pancreatic carcinoma CAPAN-2. Untreated cells were stained with anti-CD40 (BB20), anti-LT- $\beta$ -R (BDA8), or a control mAb (MOPC 21). Control antibody staining is the curve with the lowest staining in each panel.

capable of cross-linking the receptors in the right orientation will generate receptor aggregates that can signal. Dimerization of the TNF-R55 may be sufficient to trigger TNF signaling; however, in the Fas receptor case, larger aggregates need to be formed (54). When immobilized on a plastic surface, all the anti-LT- $\beta$ -R mAbs were able to induce death, including those that blocked ligand binding. Similar patterns were shown for anti-Fas receptor mAbs (51). In contrast, the anti-LT- $\beta$ -R mAbs were not very effective in solution unless mAbs to two different epitopes were mixed together. In this case, it can be envisioned that aggregates larger than dimers could form, resulting in more productive signaling. When combined with the LT- $\alpha_1/\beta_2$  ligand, some antibodies blocked activity, presumably by directly blocking the binding site, e.g., BDA8 and AGH1, although there was some evidence for mixed agonistic/antagonistic effects even in this case. At the other end of the spectrum, some mAbs, e.g., CDH10 and BKA11, potentiated the killing induced by ligand. Again, cross-linking of the receptor into small aggregates presumably facilitates the ability of ligand to productively cross-link and signal. Interestingly, the synergistic effect of the anti-LT- $\beta$ -R mAbs with the LT- $\alpha_1/\beta_2$  ligand also occurred between anti-LT- $\beta$ -R mAbs and TNF. We do not have any evidence that the TNF receptors are involved in LT- $\beta$ -R signaling, and



**Figure 6.** Growth of the human adenocarcinoma WiDr tumor in SCID mice. *A* shows the size of tumors 30 d after inoculation. Mice were treated on days 0 and 1 with saline, IFN- $\gamma$  alone, an anti-LT- $\beta$ -R mAb CBE11 with and without IFN- $\gamma$ , and a control anti-human LFA3 mAb IE6 with IFN- $\gamma$ . Animals treated with CBE11 or 1E6, represented by circles with dots, received 10  $\mu$ g/mouse per injection of antibody instead of 50  $\mu$ g for the open-circle animals. The mean of each group is indicated by a crossbar. Means, standard deviations, and (number of animals) for the five groups (left to right) were  $0.88 \pm 0.59$  (14),  $1.21 \pm 0.7$  (21),  $0.041 \pm 0.052$  (16),  $0.11 \pm 0.1$  (12) and  $0.98 \pm 1.16$  (12). *B* shows the growth of WiDr tumors as per *A*, however, the tumors were grown to an average diameter of 0.53 cm (0.076 cc) without any treatment, then intraperitoneal injections were started on day 15 and continued as indicated by the arrows. Animals were treated with IFN- $\gamma$  alone ( $\square$ ), IFN- $\gamma$  with 50  $\mu$ g 1E6 anti-LFA-3 ( $\circ$ ), or IFN- $\gamma$  with 50  $\mu$ g CBE11 anti-LT- $\beta$ -R ( $\Delta$ ). Means and standard deviations are indicated for groups of 12 animals.

therefore we interpret the effects on TNF signaling as resulting from more complex priming-like events at the intracellular level. Anti-LT- $\beta$ -R mAbs can trigger NF- $\kappa$ B activation without inducing cell death (MacKay, F., manuscript submitted for publication), thus it is possible that both the LT- $\beta$ -R and TNF-R intracellular signal transduction pathways utilize some common elements resulting in synergistic cross talk. Along with the soluble ligands and LT- $\beta$ -R-Fc forms, the anti-LT- $\beta$ -R mAbs are good tools for either activating or inhibiting the LT- $\beta$ -R signaling pathway.

TNF can induce necrosis, apoptosis, or mixed mechanisms, depending on the cell line (55, 56), whereas Fas-triggered death is generally described as occurring by apoptosis. TNF can have either fast or slow effects on cells, probably reflecting multiple mechanisms (57). The experiments described here showed a lack of classical DNA laddering, although some DNA cleavage was detected using TUNEL techniques. The nuclei appear to condense, yet the cytoplasm balloons out in a manner characteristic of necrosis. It is possible that the ballooning occurs long after the death event and is observed in these assays, which are relatively long term compared with conventionally studied apoptotic events. Morphologically, the death induced by TNF, anti-Fas receptor, and LT- $\alpha_1/\beta_2$  are similar, differing only kinetically. The signaling pathways initiated in each case may be different, as shown previously for Fas and TNF receptors (58, 59). The action of TNF is often accelerated by

the addition of cycloheximide to the culture, and the HT-29 cells are no exception. In contrast, LT- $\alpha_1/\beta_2$  lacked activity in the standard short term cycloheximide-containing assay. Either the LT- $\alpha_1/\beta_2$  action required protein synthesis, or the cytotoxic events are simply too slow to manifest themselves in the short-term assay format. In the TNF case, the signaling pathways leading to death are likely to be different depending on whether or not cycloheximide is present (60). The effects of cycloheximide and the slow pace of LT- $\beta$ -R-induced death relative to TNF and Fas suggest that LT- $\beta$ -R acts via a different pathway. This concept is intriguing since the LT- $\beta$ -R lacks a canonical death domain. Either receptor cytoplasmic domains lacking the death domain can signal death, or the LT- $\beta$ -R undergoes complex ill-defined interactions with other receptors. In light of the recent observations on the slow death mediated by TNF-R75 (23, 24), which also lacks a canonical death domain, it is possible that there are other death pathways used by some TNF family members distinct from that typified by the well-studied Fas system.

The ability to induce death selectively in tumor cells is an attractive goal. The signaling mediated by the TNF family of receptors is intriguing since this is one of relatively few cases in which a normal physiological signal can induce cell death as opposed to the loss of a signal, e.g., IL-2 or IL-3 removal. The use of TNF to treat cancers was predicated on this concept even before the emergence of programmed cell death as an important physiological process. Activation of the LT- $\beta$ -R with the CBE11 mAb in vivo blocked the growth of WiDr cells inoculated into SCID mice. Since the anti-human LT- $\beta$ -R mAb cannot bind to mouse cells, the growth inhibition mechanism must directly target the tumor cell. Likewise, the exogenously added IFN- $\gamma$  can act only on the tumor cells since mouse IFN- $\gamma$  does not bind to human IFN- $\gamma$  receptors (61) and human IFN- $\gamma$  does not bind to mouse IFN- $\gamma$  receptors. Therefore, because of the lack of an absolute requirement for IFN- $\gamma$ , it remains unclear whether the in vivo effect reflects direct cytotoxicity as observed in vitro or whether other mechanisms are contributing. If other mechanisms are involved, a wider

range of tumors may be affected in vivo than would be forecast by the in vitro analyses. Generally, more primary tumors were found to be affected by TNF in in vivo models than would be surmised from the analysis of in vitro cultured tumor lines (62).

An LT- $\beta$ -R-based antitumor strategy may be important when considered in the context of recent advances. TNF, Fas, and LT- $\alpha_1/\beta_2$  are clearly cytotoxic to tumor types with mutant p53 such as HT-29 (63), and, moreover, the TNF signaling pathway in cells of fibroblastoid or epithelial origin is not sensitive to the protective effects of bcl-2 (64). Therefore, the induction of a death sequence might occur via a route that circumvents the wild-type p53 dependency of some chemotherapeutic approaches (65). A proper understanding of these signaling processes may lead to alternative strategies for controlling cancer. Clinically, isolated limb perfusion with TNF dramatically demonstrated the soundness of the approach (66), but unfortunately the systemic application of TNF was frustrated by dose-limiting toxicity resulting from activation of inflammatory cascades. Likewise, the Fas receptor is widely distributed, and anti-Fas mAbs can be very potent inducers of apoptosis; however, receptor activation also leads to rapid necrosis of normal liver cells and death in mice. This activity would certainly complicate its therapeutic application (67). The LT- $\beta$ -R is present on most transformed cell types, and its activation also presents a potential anticancer therapy. Moreover, it is likely that sufficient IFN- $\gamma$  exists in the tumor environment to synergize with LT- $\alpha_1/\beta_2$  without exogenous administration (68). Our preliminary data indicate that LT- $\alpha_1/\beta_2$  does not effectively activate primary human endothelial cells to express the VCAM or E-selectin adhesion molecules, and hamster anti-m-LT- $\beta$ -R mAbs do not cause death (Hochman, P., G. Majeau, F. Mackay, and S. Browning, manuscript submitted for publication); therefore, LT- $\beta$ -R signaling should lack TNF-type toxicity. If the physiological activity of LT- $\alpha_1/\beta_2$  is limited to more subtle regulatory effects on lymph node function, it is conceivable that an LT- $\alpha_1/\beta_2$  therapy may provide an alternative therapy for some adenocarcinomas.

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## References

- Smith, C.A., T. Farrah, and R.G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell.* 76:959-962.
- Beyaert, R., and W. Fiers. 1994. Molecular mechanisms of tumour necrosis factor-induced cytotoxicity. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 340:9-16.
- Fiers, W. 1991. Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 285:199-212.
- Tracey, K.J., and A. Cerami. 1993. Tumor necrosis factor,

other cytokines and disease. *Annu. Rev. Cell Biol.* 9:317-343.

5. Tartaglia, L.A., D.V. Goeddel, C. Reynolds, I.S. Figari, R.F. Weber, B.M. Fendly, and M.A. Palladino, Jr. 1993. Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. *J. Immunol.* 151: 4637-4641.
6. Pimentel-Muinos, F.X., J. Mazana, and M. Fresno. 1995. Bi-phasic control of nuclear factor-kappa B activation by the T cell receptor complex: role of tumor necrosis factor alpha. *Eur. J. Immunol.* 25:179-186.
7. Paul, N.L., and N.H. Ruddle. 1988. Lymphotoxin. *Annu. Rev. Immunol.* 6:407-438.
8. Browning, J.L., A. Ngam-ek, P. Lawton, J. DeMarinis, R. Tizard, E.P. Chow, C. Hession, G.B. O'Brine, S.F. Foley, and C.F. Ware. 1993. Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell.* 72:847-856.
9. Browning, J.L., I. Dougas, E.A. Ngam, P.R. Bourdon, B.N. Ehrenfels, K. Miatkowski, M. Zafari, A.M. Yampaglia, P. Lawton, W. Meier, et al. 1995. Characterization of surface lymphotoxin forms. Use of specific monoclonal antibodies and soluble receptors. *J. Immunol.* 154:33-46.
10. Androlewicz, M.J., J.L. Browning, and C.F. Ware. 1992. Lymphotoxin is expressed as a heteromeric complex with a distinct 33-kDa glycoprotein on the surface of an activated human T cell hybridoma. *J. Biol. Chem.* 267:2542-2547.
11. Ware, C.F., P.D. Crowe, M.H. Grayson, M.J. Androlewicz, and J.L. Browning. 1992. Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells. *J. Immunol.* 149:3881-3888.
12. Ware, C.F., T.L. VanArsdale, P.D. Crowe, and J.L. Browning. 1995. The ligands and receptors of the lymphotoxin system. *Curr. Top. Microbiol. Immunol.* 198:175-218.
13. Abe, Y., A. Horiuchi, Y. Osuka, S. Kimura, G.A. Granger, and T. Gatanaga. 1992. Studies of membrane-associated and soluble (secreted) lymphotoxin in human lymphokine-activated T-killer cells in vitro. *Lymphokine Cytokine Res.* 11:115-121.
14. Crowe, P.D., T.L. VanArsdale, B.N. Walter, C.F. Ware, C. Hession, B. Ehrenfels, J.L. Browning, W.S. Din, R.G. Goodwin, and C.A. Smith. 1994. A lymphotoxin-beta-specific receptor. *Science (Wash. DC)*. 264:707-710.
15. De Togni, P., J. Goellner, N.H. Ruddle, P.R. Streeter, A. Fick, S. Mariathasan, S.C. Smith, R. Carlson, L.P. Shornick, J. Strauss-Schoenberger, J.H. Russell, R. Karr, and D.D. Chaplin. 1993. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science (Wash. DC)*. 264:703-706.
16. Banks, T.A., B.T. Rouse, M.K. Kerley, P.J. Blair, V.L. Godfrey, N.A. Kuklin, D.M. Bouley, J. Thomas, S. Kanangat, and M.L. Mucenski. 1995. Lymphotoxin-alpha-deficient mice: effects on secondary lymphoid development and humoral immune responsiveness. *J. Immunol.* 155:1685-1693.
17. Miyawaki, S., Y. Nakamura, H. Suzuki, M. Koba, R. Yasumizu, S. Ikebara, and Y. Shibata. 1994. A new mutation, aly, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *Eur. J. Immunol.* 24:429-434.
18. Pfeffer, K., T. Matsuyama, T.M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P.S. Ohashi, M. Kronke, and T.W. Mak. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell.* 73:457-467.
19. Erickson, S.L., F.J. de Sauvage, K. Kikly, K. Carver-Moore, S. Pitts-Meek, N. Gillett, K.C.F. Sheehan, R.D. Schreiber, D.V. Goeddel, and M. Moore. 1994. Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature (Lond.)*. 372:560-563.
20. Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Samashima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell.* 66:233-243.
21. Trauth, R.C., C. Klas, A.M.J. Peters, S. Matzku, P. Moeller, W. Falk, K.-M. Debatin, and P.H. Krammer. 1989. Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science (Wash. DC)*. 245:301-305.
22. Nagata, S., and P. Golstein. 1995. The Fas death factor. *Science (Wash. DC)*. 267:1449-1458.
23. Sarin, A., M. Conan-Cibotti, and P.A. Henkart. 1995. Cytotoxic effect of TNF and lymphotoxin on T lymphoblasts. *J. Immunol.* 155:3716-3718.
24. Zheng, L., G. Fisher, R.E. Miller, J. Peschon, D.H. Lynch, and M.J. Lenardo. 1995. Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature (Lond.)*. 377:348-351.
25. Cleveland, J.L., and J.N. Ihle. 1995. Contenders in FasL/TNF death signaling. *Cell.* 81:479-482.
26. Funakoshi, S., D.L. Longo, M. Beckwith, D.K. Conley, G. Tsarfaty, I. Tsarfaty, R.J. Armitage, W.C. Fanslow, M.K. Spriggs, and W.J. Murphy. 1994. Inhibition of human B-cell lymphoma by CD40 stimulation. *Blood.* 83:2787-2794.
27. Smith, C.A., H.J. Gruss, T. Davis, D. Anderson, T. Farrah, E. Baker, G.R. Sutherland, C.I. Brannan, N.G. Copeland, N.A. Jenkins, et al. 1993. CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. *Cell.* 73:1349-1360.
28. Browning, M.J., P. Krausa, A. Rowan, D.C. Bicknell, J.G. Bodmer, and W.F. Bodmer. 1993. Tissue typing the HLA-A locus from genomic DNA by sequence specific PCR: comparison of HLA genotype and surface expression on colorectal tumor cell lines. *Proc. Natl. Acad. Sci. USA.* 90:2842-2845.
29. Miller, G.T., P.S. Hochman, W. Meier, R. Tizard, S.A. Bixler, M.D. Rosa, and B.P. Wallner. 1993. Specific interaction of lymphocyte function associated antigen-3 with CD2 can inhibit T cell responses. *J. Exp. Med.* 178:211-222.
30. Browning, J., and A. Ribolini. 1989. Studies on the differing effects of tumor necrosis factor and lymphotoxin on the growth of several human tumor lines. *J. Immunol.* 143:1859-1867.
31. Crowe, P.D., T.L. VanArsdale, B.N. Walter, K.M. Dahms, and C.F. Ware. 1994. Production of lymphotoxin (LT alpha) and a soluble dimeric form of its receptor using the baculovirus expression system. *J. Immunol. Methods.* 168:79-89.
32. John, B., M. Gadnell, and K. Hansen. 1993. Epitope mapping and binding kinetics of monoclonal antibodies studied by real time biospecific interaction analysis using surface plasmon resonance. *J. Immunol. Methods.* 160:191-198.
33. Lee, S.H., B.B. Aggarwal, E. Rinderknecht, F. Assisi, and H. Chiu. 1984. The synergistic anti-proliferative effect of gamma-interferon and human lymphotoxin. *J. Immunol.* 133:1083-1086.
34. Dealy, G.B., M.S. Naylor, W. Fiers, and F.R. Balkwill. 1987. DNA fragmentation and cytotoxicity caused by tumor necrosis factor is enhanced by interferon-gamma. *Eur. J. Immunol.* 17:689-693.
35. Yonehara, S., A. Ishii, and M. Yonehara. 1989. A cell-killing monoclonal antibody (anti-fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* 169:1747-1756.
36. Ashkenazi, A., S.A. Marsters, D.J. Capon, S.M. Chamow,

I.S. Figari, D. Pennica, D.V. Goeddel, M.A. Palladino, and D.H. Smith. 1991. Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesin. *Proc. Natl. Acad. Sci. USA.* 88:10535–10539.

37. Loetscher, H., R. Gentz, M. Zulauf, A. Lustig, H. Tabuchi, E.J. Schlaeger, M. Brockhaus, H. Gallati, M. Manneberg, and W. Lesslauer. 1991. Recombinant 55-kDa tumor necrosis factor (TNF) receptor. Stoichiometry of binding to TNF alpha and TNF beta and inhibition of TNF activity. *J. Biol. Chem.* 266:18324–18329.

38. Goh, C.R., C.S. Loh, and A.G. Porter. 1991. Aspartic acid 50 and tyrosine 108 are essential for receptor binding and cytotoxic activity of tumour necrosis factor beta (lymphotoxin). *Protein Eng.* 4:785–791.

39. Oberhammer, F., J.W. Wilson, C. Dive, I.D. Morris, J.A. Hickman, A.E. Wakeling, P.R. Walker, and M. Sikorska. 1993. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:3679–3684.

40. Dusenbury, C.E., M.A. Davis, T.S. Lawrence, and J. Maybaum. 1991. Induction of megabase DNA fragments by 5-fluorodeoxyuridine in human colorectal tumor (HT29) cells. *Mol. Pharmacol.* 39:285–289.

41. Bortner, C.D., N.B.E. Oldenburg, and J.A. Cidlowski. 1995. The role of DNA fragmentation in apoptosis. *Trends Cell Biol.* 5:21–26.

42. Ledbetter, J., L.S. Grosmaire, D. Hollenbaugh, A. Aruffo, and S.G. Nadler. 1995. Agonistic and antagonistic properties of CD40 mAb G28-5 are dependent on binding valency. *Circ. Shock.* 44:67–72.

43. Raitano, A.B., P. Scuderi, and M. Korc. 1991. Upregulation of interferon-gamma binding by tumor necrosis factor and lymphotoxin: disparate potencies of the cytokines and modulation of their effects by phorbol ester. *J. Interferon Res.* 11:61–67.

44. Lynch, D. 1995. Biology of Fas. *Circ. Shock.* 44:63–66.

45. Crispe, I.N. 1994. Fatal interactions: Fas-induced apoptosis of mature T cells. *Immunity.* 1:347–349.

46. Horiuchi, A., Y. Abe, M. Miyake, K. Kimura, Y. Hitsumoto, N. Takeuchi, and S. Kimura. 1994. Role of membrane-associated lymphotoxin (mLT) in the killing activity of lymphokine-activated killer (LAK) cells towards various tumour cell lines. *Clin. Exp. Immunol.* 96:152–157.

47. Henkardt, P.A. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity.* 1:343–346.

48. Banner, D.W., A. D'Arcy, W. Janes, R. Gentz, H.J. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer. 1993. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell.* 73:431–445.

49. Engelmann, H., H. Holtmann, C. Brakebusch, Y.S. Avni, I. Sarov, Y. Nophar, O. Hadas, O. Leitner, and D. Wallach. 1990. Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *J. Biol. Chem.* 265: 14497–14504.

50. Shalaby, M.R., A. Sundan, H. Loetscher, M. Brockhaus, W. Lesslauer, and T. Espevik. 1990. Binding and regulation of cellular functions by monoclonal antibodies against human tumor necrosis factor receptors. *J. Exp. Med.* 172:1517–1520.

51. Alderson, M.R., T.W. Tough, S. Braddy, T. Davis-Smith, E. Roux, K. Schooley, R.E. Miller, and D.H. Lynch. 1994. Regulation of apoptosis and T cell activation by Fas-specific mAb. *Int. Immunity.* 6:1799–1806.

52. van Lier, R.A.W., M.O. Pool, P. Kabel, S. Mous, F. Terpstra, M.A. De Rie, C.J. Melief, and F. Miedema. 1988. Anti-CD27 monoclonal antibodies identify two functionally distinct populations within the CD4<sup>+</sup> T cell subset. *Eur. J. Immunol.* 18:811–816.

53. Gordon, J., M.J. Millsum, G.R. Guy, and J.A. Ledbetter. 1988. Resting B lymphocytes can be triggered directly through the CDw40 (Bp50) antigen. *J. Immunol.* 140:1425–1430.

54. Dhein, J., P.T. Daniel, B.C. Trauth, A. Oehm, P. Moller, and P.H. Krammer. 1992. Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 cell surface antigens. *J. Immunol.* 149:3166–3173.

55. Laster, S.M., J.G. Wood, and L.R. Gooding. 1988. Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. *J. Immunol.* 141:2629–2634.

56. Fady, C., A. Gardner, F. Jacoby, K. Briskin, Y. Tu, I. Schmid, and A. Lichtenstein. 1995. Atypical apoptotic cell death induced in L929 targets by exposure to tumor necrosis factor. *J. Interferon Cytokine Res.* 15:71–80.

57. Reid, T.R., F.M. Tortis, and G.M. Ringold. 1989. Evidence for two mechanisms by which tumor necrosis factor kills cells. *J. Biol. Chem.* 264:4583–4589.

58. Wong, G.H.W., and D.V. Goeddel. 1994. Fas antigen and p55 TNF receptor signal apoptosis through distinct pathways. *J. Immunol.* 152:1751–1755.

59. Grell, M., P.H. Krammer, and P. Scheurich. 1994. Segregation of APO-1/Fas antigen and tumor necrosis factor receptor mediated apoptosis. *Eur. J. Immunol.* 24:2563–2566.

60. Reid, T., P. Louie, and R.A. Heller. 1994. Mechanisms of tumor necrosis factor cytotoxicity and the cytotoxic signals transduced by the p75 tumor necrosis factor receptor. *Circ. Shock.* 44:84–90.

61. Peska, S., J.A. Langer, K.C. Zoon, and C.E. Samuel. 1987. Interferons and their actions. *Annu. Rev. Biochem.* 56:727–777.

62. Fiers, W. 1991. Tumor necrosis factor, characterization at the molecular, cellular and in vivo level. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 285:199–212.

63. Park, D.J., H. Nakamura, A.M. Chumakov, J.W. Said, C.M. Miller, D.L. Chen, and H.P. Koeffler. 1994. Transactivational and DNA binding activities of endogenous p53 in p53 mutant cell lines. *Oncogene.* 9:1899–1906.

64. Vanhaesebroeck, B., J.C. Reed, V.D. De, J. Grooten, T. Miyashita, S. Tanaka, R. Beyaert, R.F. Van, and W. Fiers. 1993. Effect of bcl-2 proto-oncogene expression on cellular sensitivity to tumor necrosis factor-mediated cytotoxicity. *Oncogene.* 8:1075–1081.

65. Lowe, S.W., H.E. Ruley, T. Jacks, and D.E. Housman. 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell.* 74:957–967.

66. Lejeune, F., D. Lienard, A. Eggermont, H. Schraffordt, B. Kroon, J. Gerain, F. Rosenkaimer, and P. Schmitz. 1994. Clinical experience with high dose tumor necrosis factor alpha in regional therapy of advanced melanoma. *Circ. Shock.* 43:191–197.

67. Ogasawara, J., F.R. Watanabe, M. Adachi, A. Matsuzawa, T. Kasugai, Y. Kitamura, N. Itoh, T. Suda, and S. Nagata. 1993. Lethal effect of the anti-Fas antibody in mice. *Nature (Lond.)*. 364:806–809.

68. Dighe, A.S., E. Richards, L.J. Old, and R.D. Schreiber. 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN $\gamma$  receptors. *Immunity.* 1:447–456.

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